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Adenosine 3',5'-Cyclic Monophosphate Extracted from Local Isolate of *Bacillus* Species

TARIK MOHAMMED ALI RAJAB[1], GAYLANY H. ABDULLAH[2], HAMID GHAFFOORI HASAN[1] and ABDUL AMIR H. KADHUM[3,*]

[1]Department of Chemistry, College of Education (Ibn AlHaitham), Baghdad University, Baghdad, Iraq
[2]Department of Chemistry, Faculty of Science and Health, Koya University, Koysinjaq, Arbil, Iraq
[3]Chemical and Process Engineering, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi Selangor, Malaysia

*Corresponding author: E-mail: bilal.chem.82@gmail.com

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Bacillus sp has been isolated locally from Sulaimani soil sample using nutrient agar plates. The isolate was identified as *Bacillus* genus according to the morphological feature of the colonies and cells. The bacterial growth was obtained using nutrient broth at 37 ºC of pH 7.2. Adenosine 3',5'-cyclic monophosphate was extracted from the broth medium using incubation periods of 6, 12, 18 and 24 h. The extract was purified using Dowex-50 H from column chromatography technique. The purified adenosine 3',5'-cyclic monophosphate was identified using UV, HPLC and TLC analysis.

Key Words: Adenosine 3',5'-cyclic monophosphate, Bacteria, *Bacillus species*, Column chromatography.

INTRODUCTION

Adenosine 3',5'-cyclic monophosphate (cAMP) is essentially ubiquitous and it is found in animal cells[1], plants[2], fungi[3] and bacteria[4]. In vertebrate, synthesis of cAMP is stimulated by peptide hormones interacting with protein receptors in the cell membrane. In higher cells, cAMP is degraded by cyclic nucleotide phosphodiesterases and intracellular level can decrease rapidly[5]. In enteric coliforms the intracellular concentration of cAMP is rigorously controlled[6]. Adenylate cyclase activity is controlled through a mechanism that senses environmental conditions. This signal is mediated by transport proteins located in the cell membrane. The nucleotide is degraded by cAMP phsphodiesterase; it is also readily excreted[7]. The most familiar role for cAMP in vertebrates is in the regulation of glycogen and triglyceride metabolism[1]. cAMP in bacteria functions not as an activator of the protein kinase[8], but rather as an activator of CRP, an allosteric DNA-regulatory protein[9,10] that modulates the transcription of several genes[4] and function most dramatically to regulate the expression of inducible catabolic operons. In normal situations, some cAMP is always present; even cells grown in the presence of glucose produce detectable levels of the nucleotide[11]. Cyclic nucleotides have been detected in a variety of bacteria[12]. The effect of exogenous cAMP is not always unequivocal proof of a role for the nucleotide. It usually takes high concentration of the nucleotide, three orders of magnitude greater than that found in any cells, to have any effect. The effect could be a nonspecific to the nucleotide[4]. It should be noted that cAMP has been found in *B. subtilis*[13]. A report definitively shows that cAMP is made in appreciable quantities when cells are grown with limiting oxygen[14,15]. There is another report showing that cAMP is not found in *Clostridium perfringens*[16]. Table-1 lists some bacteria in which cyclic nucleotides have been found[4].

Criteria: A, glucose-mediated repression of enzyme synthesis; B, cAMP-mediated stimulatory effects; C, cyclic nucleotide measurement; D, genetic evidence, apparent cya and crp mutants; E, effects of *in vitro* gene expression; TCA, tricarboxylic acid.

The present study represents first investigation of adenosine 3',5'-cyclic monophosphate from local isolate of *Bacillus sp* using nutrient broth medium. Also, in the present work, initially an attempt was made to partially purify the cAMP present in the culture filtrate obtained from fermentation action by *Bacillus sp.* UV, HPLC and TLC analysis were done to obtain additional data towards understanding its properties.

EXPERIMENTAL

Isolation and identification: The simplest technique that enriches for aerobic spore formers was used[17]. Different soil samples were dried in an oven at 50 ºC for overnight; a diluted soil sample was pasteurized at 80 ºC for 15 min and then plated onto nutrient agar plates. The plates were incubated at 37 ºC for 24 h and up to several days. The plates were examined after 24 h for typical *Bacillus* colonies identified as catalase-positive, gram-positive, endospore-forming rods[17,18].

TABLE-1
REPORTS OF CYCLIC NUCLEOTIDES
IN MISCELLANEOUS BACTERIA

Microorganism	Property	Criteria
Aeromonas spp	"Suicide" growth on glucose	A, B
Archaebacteria	Measurable cAMP	C
Arthrobacter oxidans	Glucose repression of 6-hydroxynicotine oxidase	A
Bacillus circulans	β-Xylanase expression	B
Bacillus subtilis	cGMP and sporulation	B
	measurable cAMP in O2-limited cells	C
Chlamydia trachomatis	Development	B
Erwinia chrysanthemi	Pectate lyase expression	A, B
Klebsiella aerogenes	*hut* operons induction	B, E
Klebsiella pneumoniae	TCA cycle enzymes	A, B
	Nitrogen metabolism	A, B
Legionella pneumophila	Cell growth	B
Mycobacterium smegmatis	Fatty acid synthesis	B
Mycoplasma pneumoniae	Glass attachment	B
Pseudomonas fluorescens	Antibiotic production	A, B
Vibrio cholera	–	A, B
Vibrio fischeri	Bioluminescence	B, C, D
Vibrio parahaemolyticus	Protease production	D
Marine microorganisms	cAMP uptake	C

Extraction of cAMP from culture broth: Nutrient broth in 250 mL flasks were used for detecting cAMP. The flasks were inoculated with 1 mL/flask from local isolate of *Bacillus sp*, in which it contain 11×10^7 cell/mL, then the flasks were incubated at 37 ºC for different incubation times (6, 12, 18 and 24 h). The broth culture after each incubation time was centrifuged in (NUVE NF 615) at 5000 rpm for 15 min. A protocol of Brooker[19] was adopted so that cAMP was isolated in the supernatant. The procedure was used by taking 3 mL of centrifuged broth sample to the test tube and then addition of 0.4 mL of 5 % $ZnSO_4$ (5 g of zinc sulphate dissolved in 100 mL distilled water) was followed, then 0.4 mL of 0.3 N $Ba(OH)_2$ (4.725 g of dissolved in 100 mL distilled water) was added. Mixed well and then centrifuged for 10 min at the 3000 rpm, this would precipitate protein, phosphates, pyrophosphates and all nucleotides except cAMP[19-21].

Column chromatography purification: The technique was used to purify the extracted cAMP; a volume of 3 mL of the supernatant (containing cAMP) from step 2 was loaded on a Dowex 50W hydrogen form of the dimension (1 cm × 5 cm) and elutated with distilled water at flow rate of 3 mL/min. The eluted fractions of 3 mL were collected[21] (Fig. 1). After the collection of 15 fractions, it was found that fraction No. 2 contains the highest activity of the purified cAMP as shown in the Fig. 1. Same results were obtained in which fraction No. 2 represents the activity of the purified cAMP peak.

UV analysis of cAMP: cAMP has a strong characteristic UV spectrum with a maximum λ at 258 nm at pH 7.

To prepare standard solution, *ca.* 1 mg of cAMP was dissolved in 25 mL distilled water, the pH was adjusted to 7, where 3 mL of cuvette has been used as a sample container and after the appropriate dilution, scanned the absorbance using (Helios alpha type) ultraviolet spectrophotometer[22].

Absorption at neutral pH: After neutralization the standard solution, 3 mL of the standard cAMP solution was

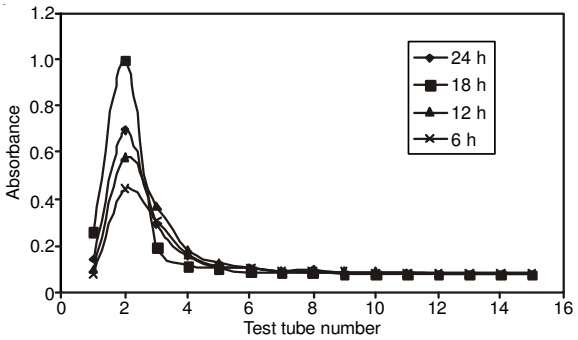

Fig. 1. Column purification of extracted cAMP from *Bacillus S*. at different time using Dowex 50 w (1 cm × 5 cm) colum

scanned by spectrophotometer between 220-300 nm. A maximum absorbance at 258 nm was obtained. The same protocol was applied for extracts of cAMP. In which fraction No. 2 contained separated and purified cAMP and reflected a sharp peaks at λ = 258 nm[23] (Fig. 2).

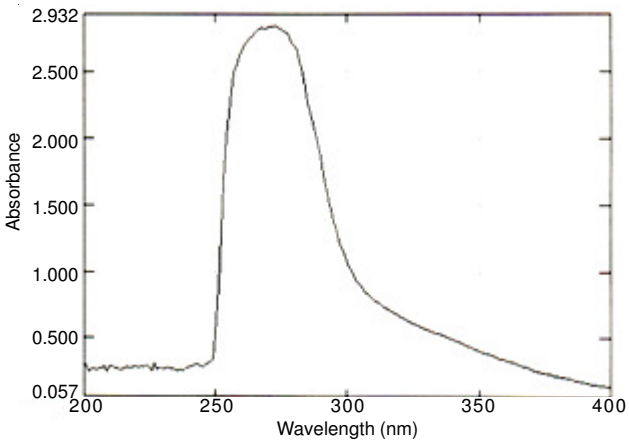

Fig. 2. Spectrum of the authenticated cAMP shows maxima at 258 nm

Absorption at acidic and basic pH: Three mL of standard cAMP solution and same volumes of extracted cAMP acidified at pH = 2 by the addition of 1N HCl. These fractions were scanned using spectrophotometer between 220-300 nm. Maximum absorbance was obtained at the wave length of 256 nm (Fig. 3). Same protocol was used but the fractions were made basic at pH = 12 using 1 N NaOH and scanned using spectrophotometer between the 220-300 nm. Maximum absorbance was obtained at wavelength of 260 nm (Fig. 4)[23,24].

HPLC analysis: HPLC 10AVP consist of two deliver pumps, 10 AVP controller, the injection was Reodyne 7125 USA, the eluted chromatogram was monitored by UV-VIS 10 AV-SPD Detector. The separation was performed on a reversed phase (250 mm × 4.6 mm h.d). Column 5 μm particle size, type DB-C_{18}. Mobile phase: methanol: water 50/50 (v/v). Flow rate: 1 mL/min. Temperature: 25 ºC. Detection: UV set at 260 nm. Firstly 5 μL of mobile phase without standard and samples was injected to comparing with another chart. The standard was tested by taking 1 mL of standard and added to 1 mL of mobile phase. After mixing in vortex, 5 μL was injected into HPLC and by the detector recording the chromatogram chart and measuring the retention time. Same technique was used for determination the retention time of the purified cAMP eluted peak (step 3)[25,26].

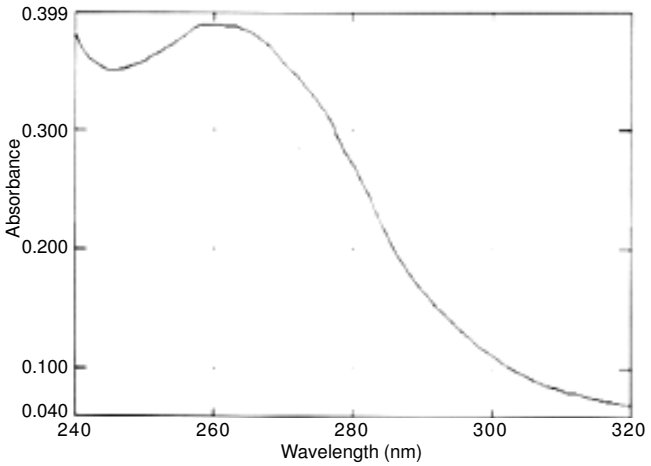

Fig. 3. UV Spectrum of the extracted cAMP at 256 nm using acidic medium

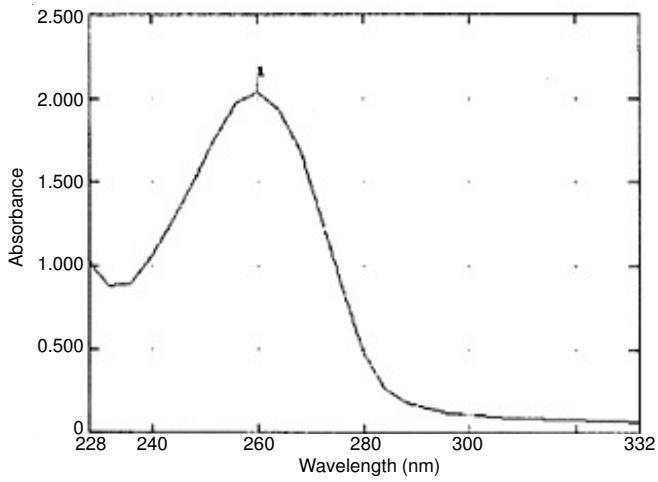

Fig. 4. UV Spectrum of the extracted cAMP a 260 nm using basic medium

TLC analysis: A uniform thin layer silica gel G60 has been used as the stationary phase in the project to separated cAMP. Silica gel G60 was mounted at the thickness of 300 µm on the glass plate and the area of (20 cm × 20 cm). The silica gel G60 plate was kept at 100 ºC for 1 h for reactivation before the application of samples.

Sample preparation: The extracted cAMP (fraction 2) was evaporated to dryness under vacuum at 55 ºC and cooled after dryness then mixed with 20 µL distilled water[25].

Sample application: A micropipette was used for applying spotting 2 (cm) from the edge of the plate. Typical spot sizes were 5 µL. Drying of the spots was achieved using an air blower; more samples were then applied if necessary.

Plate development: Development of the plate was achieved in a glass tank in which the inside wall has been covered by filter-paper (Whatman No. 1) in order to ensure equilibration after saturation of the atmosphere within the tank by the solvent vapour. Developing solvent was added to a depth of *ca.* 1.5 cm of the tank. The tank top was covered to reach the equilibrium within 1 h and the plate was then placed vertically in the tank.

Spot detection: Ultraviolet light absorbing spots were visualized by spraying with 0.01 % ethanolic solution of 2,7-dichlorofluresceine; this was used for nucleotides, cyclic nucleotides which appeared as dark areas on a fluorescent background. Several solvent systems were used, but *n*-butanol: glacial acetic acid:distilled water; 2:5:3 (v/v) was the major system used as previously suggested[27,28]. The R_f values for the standard and the purified cAMP were recorded and given in Table-1.

RESULTS AND DISCUSSION

Identification of cAMP using UV analysis: Fig. 1 shows the purification scheme analysis of cAMP peak from different time of incubation. Incubation for 18 h reflects the highest activity of cAMP. It is known that cyclic adenosine 3',5'-nucleotides contains hetero nitrogenous bases and the basic principle of UV analysis for such type of compounds depends on the presence of such nitrogenous bases[29,30]. As cAMP and other derivatives contain an adenosine base which is a conjugated ring system, these compound have a strange characteristic UV spectrum with a maximum wavelength of 258 nm at pH 7.0 (Fig. 2)[30]. The UV analysis of the purified cAMP from *Bacillus S.* was performed at different pH values as shown in Figs. 3 and 4. Shifting in the wavelength of the peak absorbance has been occurs. Regular use of this property was made in identification and quantification. These results were similar to those previously described[23] in which cAMP fractions were purified from different tissues. The UV spectrum of the *Basillus S.* was compared with the spectra of the standerd cAMP. This is in consistence with the findings of Krstulovic *et al.*[28]. No interferences were seen from other naturally occurring constituents in the gradients elution separation of the cAMP extract. The analysis does not require any pre-concentration and its sensitivity is of considerable importance in analyzing limited amounts of samples for the exceedingly low concentration of this compound. The alternative isocratic elution mode optimized for the selective analysis of cAMP, offers a rapid and reliable method for determination of this important compound in biological samples. The method of analysis employed in this study lend themselves easily to several simple identification methods , which are necessary if the identity of the components of complex mixtures of biological compounds is to be determined valuably and unambiguously. This method coupled with an appropriate sample preparation can be used for biological samples of different origin.

Identification of cAMP using TLC analysis: Thin layer chromatography analysis has been routinely used for the analysis and identification of cAMP and its derivative, in which the R_f values used for comparison between standard and extracted *Basillus S.* cAMP (Table-2). The chances that chromatographic peak is a compound other than cAMP seem remote. Bases or nucleosides would be eliminated by the other extractions. The zinc-barium precipitation, the Dowex-50W column and by thin layer chromatography were used for this purpose. Similar results were reported by Kuo *et al.*[31] and Brooker[25].

TABLE-2
TLC-ANALYSIS FOR STANDARD CAMP AND NORMAL AND LEUKAEMIC URINAY CAMP

Parameter	R_f Value
St. CAMP	0.44
UN. CAMP	0.43
UL. CAMP	0.45

Identification of cAMP using HPLC technique: High performance liquid chromatography is the most versatile and widely used type of elution chromatography, the technique is used by chemists to separate and determine a variety of organic, inorganic and biological materials[30]. The values of retention time 3.02 min, confirm the successful extraction of both standard and purified cAMP extracted from *Bacillus S*. Fig. 5. The retention times obtained in this study differed from that previously reported by Newton[24], Brooker[25] and Krustolovic[28], whose findings also show different retention times. These variations can be explained by differences in the type of HPLC model, length of column, types of mobile phase and its flow rates and the type of packed column material[32]. Data obtained from the three techniques used for the analysis and identification of cAMP provide strong evidence for the similarity between the standard and extracted cAMP, despite slight differences between them, which we believe may be due to differences in purity, sensitivity of the instruments and methods used.

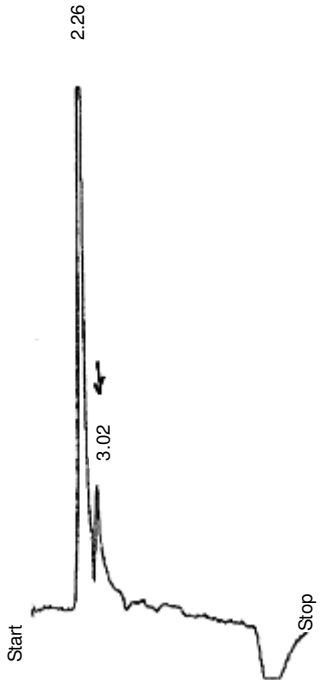

Fig. 5. HPLC analysis for cAMP peak from isolated *Bacillus* species

Conclusion

It seems that cAMP signaling is involved in most aspects of differentiation and maturation of cells. As it was recognized that cAMP has a significant role in the regulation of carbohydrates and lipids regulation through gene regulation mostly involved in energy metabolism. Thus, cAMP can be play a significant role in causing a conformational changes in cell through its cAMP receptor protein. Thus, the aim of this study was focused on the bacteria cell growth secretion of cAMP in a simple assay. The advantages of the procedure described above seems significant. The sensitivity of the assay is high and the actual assay is simple to perform.

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