A NOVEL METHOD FOR THE PREPARATION OF ADENOSINE 3'5' CYCLIC PHOSPHATE LABELLED WITH ^{14}C OR ^{3}H

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SUMMARY

High yields of cyclic AMP (cAMP) and cyclic GMP (cGMP) could be obtained by near quantitative conversions of adenine and guanine by aerobically-grown non-proliferating cells of Corynebacterium murisepticum. The procedure is especially suited for the preparation of ^{14}C or ^{3}H labelled cAMP and cGMP from respective purine base precursors.

Key words - Adenine, cyclic AMP, cyclic GMP, Hypoxanthine, Corynebacterium murisepticum

INTRODUCTION

Adenosine 3'5' cyclic phosphate (cAMP) labelled with ^{14}C or ^{3}H is a useful radioactive biochemical especially for the assay of cAMP level in the biological fluids by competitive protein binding analysis. The procedure for the chemical synthesis of radioactively labelled cAMP involves complex and multistep operations (1). The starting material is labelled AMP which itself is prepared chemically in several steps from labelled adenine (2,3). The enzymatic method for the preparation of labelled cAMP from labelled ATP is commercially unsuitable for large scale preparations since it requires either purified ATPase-free adenyl cyclase or crude adenyl cyclase with ATP regenerating system (4).

We have developed a simple, efficient and inexpensive procedure for the preparation of labelled cAMP from labelled adenine using nonproliferating cells of Corynebacterium murisepticum. This bacterium converts purine bases to their respective nucleoside 3'5' cyclic phosphates through the salvage pathway (5,6). The advantage of this procedure lies in the fact that it neither involves multistep operations nor does it require expensive materials like labelled ATP and purified enzymes.

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EXPERIMENTAL

Corynebacterium murisepticum ATCC 21374 was maintained on nutrient agar slants. The inoculum medium was made of (w/v) 2% glucose, 1% yeast extract, 1% peptone and 0.3% NaCl (pH 7.3). The growth medium for obtaining the cells was composed of (w/v) 0.2% KH_2PO_4, 0.6% K_2HPO_4, 0.3% $MgSO_4 \cdot 7H_2O$, 1% peptone, 1% yeast extract, 0.3% NaCl and 2% glucose (sterilized separately). The pH was adjusted to 7.8 by 2N KOH.

For cultivation of the cells, a loopful of culture grown on an agar slant was transferred to 50 ml of inoculum medium in a 250 ml Erlenmeyer flask and incubated at 30° for 24 hours on a rotary shaker (200-250 rpm). Two ml of this inoculum culture were transferred to 100 ml of the growth medium in a 500 ml Erlenmeyer flask and incubated as above for 12-24 hours. The exponentially growing cells were harvested by centrifugation at refrigerated temperature (0-4°) and washed once with an equal volume of ice-cold saline. The washed cell pellets were stored at -70°.

Adenine-8-^{14}C (specific activity 50.8 µCi/µmole) and adenine-8-^{3}H (specific activity 4.1 mCi/µmole) were obtained from the Isotope Division of this Research Centre. Synthesis of labelled cAMP from labelled adenine by washed cells was studied in a reaction mixture containing $MgSO_4$, 50mM; potassium phosphate buffer pH 7.5, 100mM; glucose, 50mM; ^{3}H or ^{14}C-labelled adenine, 1-10mM and approximately 2.5×10^{10} cells per ml. The complete reaction mixture was incubated at 30° with vigorous stirring. After 2-3 hours of incubation, the mixture was centrifuged and the supernatant together with the pooled cell washings was adjusted to pH 2 with 2N HCl and passed through an activated charcoal column (4x1.5 cm). The column was washed thoroughly with water and the labelled cAMP formed was eluted either with 15% pyridine or 50% ethanol containing 1.5% NH_4OH. The eluates were dried in vacuo. The dried sample was taken up with 0.2 ml water and subjected

to preparative paper chromatography on a Whatman No. 3 paper using 1M NH_4HCO_3 or water saturated butanol - 25% NH_3 (100:1 v/v) as the solvent system. The cAMP band on the chromatogram was identified under ultraviolet light (using a chromatol portable UV lamp, Hanovia lamps), cut and eluted with water by the descending technique. The eluates were evaporated to dryness in a rotary evaporator at room temperature and rehydrated with a small volume of 50% ethanol-water and stored at -20°C. The nucleotide concentration was determined by measurement at 259 nm in a Perkin-Elmer 124 Double-Beam Spectrophotometer (5). To determine the radioactivity, an aliquot of the labelled product was spotted on a Whatman No. 1 paper strip, dried by a hot air blower, placed in a vial containing 10 ml scintillation fluor (0.5% PPO in toluene) and counted in a Beckman LS-100 Liquid Scintillation System.

RESULTS

The results of a typical experiment given in Table 1 indicate that there is almost quantitative conversion of added ^{14}C-adenine to ^{14}C-cAMP by the cells of C. murisepticum. The specific radioactivity of the isolated ^{14}C-cAMP was found to be nearly same as that of the ^{14}C-adenine. Similar results were obtained with added ^{3}H-adenine.

Table I: Synthesis of radioactively labelled cAMP from radioactively labelled adenine

Purification step	^{14}C-labelled cAMP			^{3}H-labelled cAMP		
	Total cAMP (µCi)	Radiochemical yield %	Sp. radioactivity (µCi/µmole)	Total cAMP (mCi)	Radiochem. yield %	Sp. radioactivity (mCi/µmole)
1. Supernatant	509	98	-	3.7	74	-
2. Charcoal adsorption	505	97.2	-	3.4	68	-
3. Preparative pap. chromatography	501	96.2	50.7	3.0	60.0	4.05

The reaction mixture contained either 514 µCi of adenine-8-^{14}C (~10mM) or 5 mCi of adenine-8^{3}H (~1.2mM). An aliquot of the supernatant or of the eluate from charcoal step was subjected to paper chromatography as described in the text and radioactivity in cAMP spot determined.

As seen from the chromatogram (Fig. 1) treatment of labelled reaction

product with bovine heart phosphodiesterase (7) resulted in complete disappearance of radioactivity from cAMP position and its appearance in 5' AMP position. This confirms the 3'5' phosphodiester linkage of the reaction product.

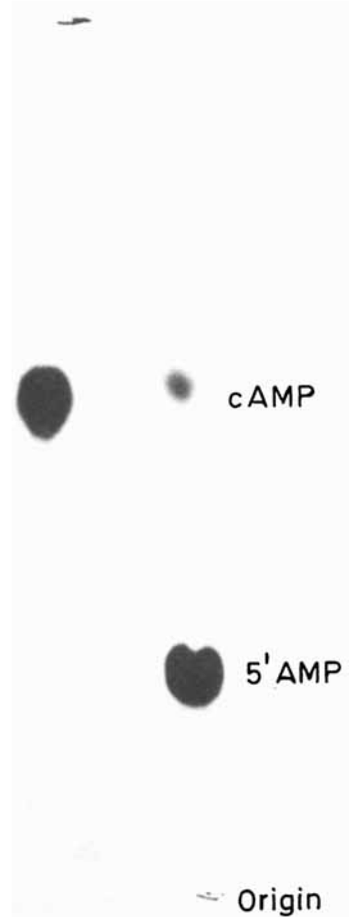
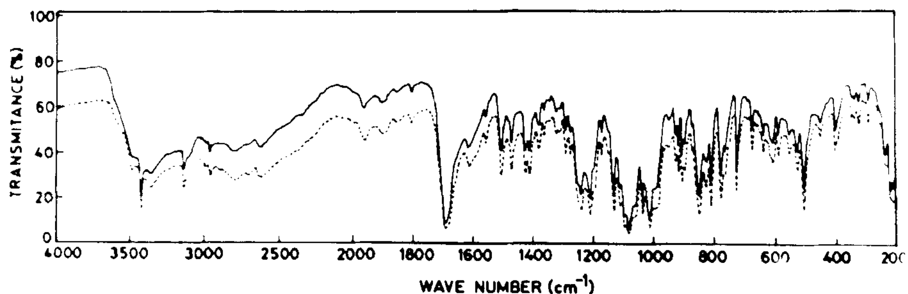


Fig. 1. Autoradiogram of bovine heart phosphodiesterase (Sigma Chemical Co. St. Louis, USA)-treated radioactive labelled material isolated from the reaction mixture, after incubation for two hours with C. murisepticum cells. An aliquot of the enzyme-treated labelled material was chromatographed on a Whatman No. 1 paper using isopropanol: 1M ammonia: 0.1M boric acid (60:10:30) as the solvent system. The chromatogram was autoradiographed using Kodak-X-ray film.

As seen in Fig. 2, the infrared spectrum of the isolated ^{14}C-labelled reaction product corresponded well with the authentic cAMP.

The Rf values obtained in paper chromatography of the isolated compound with various solvent systems were the same as those reported for the authentic cAMP (8).

Fig. 2. Infrared spectra in KBr of (i) the ^{14}C-labelled product formed by C. murisepticum cells from ^{14}C-adenine and (ii) authentic cAMP.
———Isolated, ---------Authentic



In a separate experiment, washed C. murisepticum cells incubated in the reaction mixture containing cold adenine and ^{32}P-orthophosphate under the same

conditions as above quantitatively formed ^{32}P-labelled product identified as cAMP.

It may be noted from table 1 that the radiochemical yield in ^{14}C-adenine experiment is 98% whereas that in ^{3}H-adenine experiment is 74%. The low radiochemical yield in ^{3}H-adenine can solely be attributable to the low concentration of adenine used in the reaction mixture. It was found that loss of radiochemical yield was directly related to the retention of adenine radioactivity by the cells presumably by an exchange with the cellular pool of adenylate. For bacterial cells the adenylate pool has been reported to be in the range of 1-2mM (9). This may well account for the 2% loss of radiochemical yield in ^{14}C experiment where 10mM of adenine was present in the reaction mixture and 26% loss of radiochemical yield in the ^{3}H experiment where 1.2mM of adenine was included in the reaction mixture.

During the course of investigation, it was revealed that the efficiency of cAMP formation is dependent on the manner in which C. murisepticum cells are cultivated and the incubation condition of the reaction mixture. Cells harvested from growth in stationery conditions or without proper aeration/agitation converts most of the labelled adenine to hypoxanthine. Hence vigorous aeration of the growth medium is imperative. Further it was found necessary to carry out reaction with vigorous shaking to preclude the formation of hypoxanthine.

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