^{32}P-3',5'-CYCLIC AMP: A SIMPLE PREPARATIVE PROCEDURE*

R.H. Symons

Department of Biochemistry, University of Adelaide, Adelaide,
South Australia, 5001.

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SUMMARY

A two step chemical procedure is described for the preparation of ^{32}P-3',5'-cyclic AMP with a specific activity of greater than 1 mC/µmole and with an overall yield of about 45% starting with ^{32}Pi. The first step is the preparation of ^{32}P-5'-AMP which is then converted to cyclic AMP by the action of DCC in refluxing pyridine. The product is isolated by paper chromatography. The method is applicable to the synthesis of other ribo- and deoxyribonucleoside 3',5'-cyclic phosphates.

The procedure described here is a modification and combination of two chemical methods described previously. The first step is the coupling of 1.0 µmole of ^{32}Pi (1 - 5 mC) to an excess (50 µmoles) of 2',3'-O-isopropylidene adenosine using trichloroacetonitrile as condensing agent in the presence of triethylamine and with dimethyl sulphoxide as solvent (Symons, 1966, 1968). The reaction mixture is purified by paper chromatography to give the 2',3'-O-isopropylidene 5'-AMP from which the protecting group is removed by acid hydrolysis. After conversion of the 5'-AMP to the triethylammonium salt, cyclisation is

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Abbreviation: DDC, N,N'-dicyclohexlcarbodiimide.

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achieved by refluxing for 1.5 hours under dilute conditions in anhydrous pyridine in the presence of DCC (Smith, Drummond and Khorana, 1961). The resultant 3',5'-cyclic AMP is then isolated by paper chromatography in a yield of about 45% relative to ^{32}Pi and with a specific activity of about 1 - 5 mC/μmole.

EXPERIMENTAL

Materials: Pyridine (Mallinckrodt A.R.), acetonitrile, dimethyl sulphoxide and triethylamine were dried by distillation from and storage over calcium hydride. 3',5'-Cyclic AMP and 2',3' O-isopropylidene ribonucleosides were obtained from Sigma Chemical Co. Chromatography solvent A consisted of n-propanol: conc. NH_4OH: water (55:20:25 by vol.). Before use, sheets of Whatman 3MM chromatography paper were well washed by descending chromatography in 10% acetic acid, then in Solvent A and finally in water. A simple vacuum line, similar to that described by Greenlees and Symons (1966), has been used in this work for the final drying of reaction mixtures.

^{32}P-Adenosine 5'-monophosphate: The reaction mixture for the synthesis of this nucleotide was prepared as described previously (Symons, 1966, 1968) starting with 1 μmole (1 - 5mC) of ^{32}Pi. After incubation of the reaction mixture at 37° for 30 minutes, approx. 1.0 ml of ethanol was added and the mixture dried as a band 30 cm long on a washed sheet of Whatman 3MM chromatography paper. After chromatography overnight in Solvent A, the chromatogram was thoroughly dried and the narrow band of 2',3'-O-isopropylidene 5'-AMP cut out with a guillotine and eluted with water. The solution was taken to dryness in a small round bottom, long-necked flask, 0.5 ml of 2N acetic acid was added and the stoppered flask and contents heated at 100° for one hour to remove the isopropylidene group. The acetic acid was then removed on the rotary evaporator by the addition and removal of three 1 ml portions of water.

The residue was dissolved in about 3 ml of 30% aqueous pyridine and the solution slowly passed through a column (0.8 cm diam.) containing 1 gram of well-washed Zeokarb-225 (pyridinium) resin. After washing the column with a small volume of 30% pyridine, four drops of triethylamine were added to the effluent which was taken to dryness in a 50 ml long-necked flask on the rotary evaporator. The flask contents were dried by the addition and removal of acetonitrile, twice on the rotary evaporator and finally once on the vacuum line. Dry air was let into the flask which was removed from the vacuum line and stoppered.

Chromatography of the reaction mixture prior to the removal of the isopropylidene group was necessary because, with washed papers and in contrast to unwashed papers (Symons, 1966, 1968) most of the unreacted ^{32}Pi moved away from the origin and ran with an Rf similar to that of unprotected 5'-AMP. Further, the use of washed chromatography papers plus the treatment with cation exchange resin were necessary to ensure removal of unidentified material which considerably decreased the solubility of 5'-AMP in anhydrous pyridine in the following step.

<u>^{32}P-3',5'-Cyclic AMP</u>: To the dry triethylammonium salt of ^{32}P-5'-AMP was added 0.6 ml of dimethyl sulphoxide, 5 ml of dry pyridine and about 8 μmoles (1.6 mg) of DCC in 0.1 ml of pyridine. The mixture was then heated under reflux on a hot plate for 1.5 hours with protection from moisture by a silica gel guard tube. After removal of the pyridine on the rotary evaporator, 2 ml of 50% aqueous ethanol was added and the flask contents warmed to

ensure solution of the contents. The solution was then dried as a band 30 cm long on a sheet of Whatman 3MM chromatography paper together with a marker of cyclic AMP on each side of the chromatogram. After chromatography overnight in Solvent A, the band of cyclic AMP was cut out and eluted with 0.1mM EDTA, pH 7.0, and stored at -15°. The product was obtained in an overall yield of 40 - 50% relative to ^{32}Pi and with a specific activity of 1 - 5 mC/µmole.

The identity of the product was confirmed by its chromatographic, electrophoretic and spectral properties which were identical to those of a sample of cyclic AMP. Further, after paper electrophoresis of the product at pH 8.0, 98% of the total radioactivity was associated with the single band of ultra-violet light absorbing material.

In the cyclisation step, dimethyl sulphoxide was added to aid in the solubilisation of the 5'-AMP during reflux as, in its absence, yields were somewhat lower.

Preparation of other 3',5'-cyclic nucleotides. The method described above has also been used for the preparation of 3',5'-cyclic GMP, CMP and UMP in yields of 35 - 50% relative to ^{32}Pi. For the synthesis of 3',5'-cyclic deoxynucleotides, a mixture of the 3'- and 5'-^{32}P-mononucleotides, prepared by the phosphorylation of the unprotected deoxynucleoside (Symons, 1969), was used for the cyclisation step; yields of 3',5'-dAMP, dCMP, dGMP and dTMP varied from 35 - 60% relative to ^{32}Pi.

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