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The rapid, simple and improved preparation of high specific activity α-[^{32}P]dATP and α-[^{32}P]ATP

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ABSTRACT

An improved method is described for the rapid and simple preparation of α-[^{32}P]dATP and α-[^{32}P]ATP from ^{32}Pi in good yields and with specific activities from 20 - 150 Ci/mmol. The two-step procedure involves the chemical synthesis of the mononucleotide followed by its enzymic conversion to the triphosphate with myokinase (EC 2.7.4.3) and pyruvate kinase (EC 2.7.1.40) in the presence of trace amounts of dATP or ATP to prime the reaction. The two steps are carried out in the same reaction flask and the only purification step required is a step-wise elution from a column of DEAE-cellulose.

INTRODUCTION

The α-[^{32}P]labelled ribo- and deoxyribonucleoside 5'-triphosphates are the labelled substrates of choice for many RNA and DNA polymerase reactions. Although these can be prepared by completely chemical methods (1), a more practical approach has been a two-step procedure starting with [^{32}P]-orthophosphoric acid and involving the chemical synthesis of the mononucleotide followed by its enzymic conversion to the triphosphate using either ATP or dATP as the phosphate donor (2). For the non-adenine containing nucleotides, the enzymic conversion is carried out with a partially purified nucleotide kinase preparation from Escherichia coli (3) using a several-fold molar excess of ATP or dATP (2). In the synthesis of α-[^{32}P]ATP and α-[^{32}P]dATP, the mononucleotide is readily converted to the triphosphate by the combined action of myokinase (EC 2.7.4.3) and pyruvate kinase (EC 2.7.1.40) in the presence of trace amounts of the corresponding unlabelled triphosphate to prime the reaction (2).

In this way, there is very little dilution of the specific activity of the product.

In all cases, the mononucleotide reaction mixtures are purified either by paper chromatography or column chromatography (2,4) and the triphosphate reaction mixtures by paper chromatography in an isobutyric acid solvent which very effectively separates the non-adenine containing nucleotide products from the faster running unlabelled ATP or dATP. This solvent also fractionates all the nucleotides into the mono-, di- and triphosphates (2).

Paper chromatography steps have the obvious disadvantage of a potentially high radiation exposure when handling large amounts of radioactivity. In addition, [^{32}P]triphosphates eluted from paper chromatograms must be further purified by step-wise elution from DEAE-cellulose to remove material inhibitory to polymerase reactions when the triphosphates are used without dilution by unlabelled carrier (2). Triphosphate reaction mixtures can be purified by salt gradient or step-wise elution from DEAE-cellulose or DEAE-Sephadex but the labelled non-adenine containing triphosphate will be contaminated with the unlabelled ATP or dATP used as phosphate donor (2,5). This may not be important in some polymerase reactions; e.g., in the labelling of double-strand DNA by 'nick translation' (6) using E. coli DNA polymerase I.

The previous method (2) has now been improved for the rapid and simple preparation from [^{32}P]orthophosphoric acid of α-[^{32}P]dATP and α-[^{32}P]ATP in good yield and with specific activities from 20 - 150 Ci/mmol. Higher specific activities could be readily obtained. The two-step chemical and enzymic synthesis is carried out in the same reaction flask and the only purification step required is a step-wise elution from a column of DEAE-cellulose. The product can be used directly in polymerase reaction mixtures without further purification and without dilution by the addition of unlabelled carrier. The whole procedure can be readily completed in less than three hours. The method is most appropriate for investigators who, for economic reasons, must prepare their own labelled nucleotides or for those investigators who enjoy

doing so.

MATERIALS AND METHODS

High specific activity [^{32}P]orthophosphoric acid in dilute HCl was obtained at a concentration of 20 mCi/ml from the Australian Atomic Energy Commission, Lucas Heights, New South Wales, and was used as soon as possible after arrival. Dimethyl sulphoxide, acetonitrile and trichloroacetonitrile were purified as described (1,2); the purified reagents are stable for years when stored sealed in the dark at room temperature. Myokinase (5 mg/ml in 3.2 M $(NH_4)_2SO_4$, 1 mM EDTA, pH 6), pyruvate kinase (10 mg/ml in 2.2 M $(NH_4)_2SO_4$, pH 6), 2'-deoxyadenosine and 2',3'-O-isopropylidene adenosine were obtained from Sigma Chemical Co. Polyethyleneimine (PEI) thin-layer plastic-backed plates were Macherey – Nagel Polygram CEL 300 PEI/UV_{254}.

All evaporations were carried out using a rotary evaporator connected to a water pump and with a water bath at about 40° for heating. Sterile solutions and techniques were used wherever possible to eliminate nuclease contamination of the final product. Solutions of NH_4HCO_3 were prepared just before use in sterile water. DEAE-cellulose (stored in 0.5 M NH_4HCO_3) was packed to form a 0.85 x 3.0 cm column and then washed successively with 0.1 M NaOH, water, 0.5 M NH_4HCO_3 and 0.07 M NH_4HCO_3.

For radiation protection, reaction flasks in Perspex beakers were handled with lead-impregnated gloves and all work was carried out behind Perspex screens. For further protection during the column chromatography step, a strip of lead (3.0 x 30 cm) with a hole punched in the centre was draped in the shape of a ∩ over the column.

Preparation of α-[^{32}P]dATP

About 30 - 50 μmoles of 2'-deoxyadenosine is weighed into a small reaction flask prepared from a B10 Quickfit test tube (10 cm long) with a small bulb blown in the end. The required volume of 10 mM H_3PO_4 (stored at -15°) is added followed by the ^{32}Pi in dilute HCl (see Table 1). A few drops of triethylamine are added to neutralize the HCl and

the neck of the flask is rinsed down with about 0.5 ml of ethanol. The flask contents are taken to dryness on a rotary evaporator and further dried by the evaporation of two 0.5 ml lots of acetonitrile.

To the flask is added 200 µl of dimethyl sulphoxide, 1 µl of triethylamine and 1 µl of trichloroacetonitrile and the stoppered flask is well shaken to dissolve the contents. After incubation for 30 to a maximum of 40 minutes at 37°, a few drops of ethanol are added to the pale yellow reaction mixture and all volatile material removed on the rotary evaporator (the dimethyl sulphoxide remains).

While the rotary evaporation is taking place, the following kinase reaction mix is prepared. To 0.6 ml of a solution of 20 mM KCl, 10 mM magnesium acetate, 50 mM Tris-Cl, pH 8.0, is added 25 µl of 0.1 M potassium phosphoenolpyruvate, 1 µl of 1.0 mM dATP, 1 µl 2-mercaptoethanol, 2 µl pyruvate kinase (10 mg/ml) and 2 µl myokinase (5 mg/ml). This mixture is then added rapidly to the reaction flask which is incubated at 37°. After 15 - 20 min, a small sample is removed, diluted with water and analysed by thin-layer chromatography on a 1.5 x 5 cm strip of PEI-cellulose using 0.5 M NH_4HCO_3 as solvent. Markers of dAMP and dATP are dried onto the origin prior to application of the sample. After running for about 5 min, the thin-layer is dried, the markers located under ultra-violet light and the regions corresponding to dATP, dADP and dAMP (plus the region in front of dAMP so as to include Pi) are cut out and counted by Cerenkov radiation.

Provided the kinase reaction is proceeding satisfactorily as indicated by the conversion of dAMP to dATP, the reaction mixture is diluted with 4 ml of water (usually after a total incubation time of 30 - 40 min) and run onto a 0.85 x 3.0 cm column of DEAE-cellulose (HCO_3^-), prepared as described above. The column is washed with 20 ml 0.07 M NH_4HCO_3 to elute ^{32}Pi, [^{32}P]dAMP and α-[^{32}P]dADP and then the α-[^{32}P]dATP is eluted with 10 ml 0.25 M NH_4HCO_3 into a 50 ml Quickfit flask with a medium length neck. After the addition of 1.5 ml triethylamine and 3 ml ethanol, the flask contents are taken to dryness on the rotary evaporator and then two 3 ml lots of

ethanol are added and evaporated to ensure complete removal of the triethylammonium bicarbonate. The α-[^{32}P]dATP is dissolved in 1.5 ml 0.1 mM EDTA, pH 7, and stored at -15°. The yield and purity of the product are given in Table 1.

Preparation of α-[^{32}P]ATP

The procedure is exactly the same as for the preparation of α-[^{32}P]dATP but with the following modifications:

1. 2',3'-O-Isopropylidine adenosine is used in place of the 2'-deoxyadenosine.

2. After the chemical synthesis of the [^{32}P]AMP and the addition and removal of a few drops of ethanol, 0.8 ml 0.1 N HCl is added and the stoppered flask heated in a boiling water bath for 5 min to hydrolyse the isopropylidene group. The HCl is then neutralized by the addition of several drops of triethylamine and the water removed on the rotary evaporator

TABLE 1. Synthesis of α-[^{32}P]dATP

Carrier H_3PO_4 (μmol)	^{32}Pi (mCi)	Deoxy-adenosine (μmol)	α-[^{32}P]dATP			
			mCi[a]	Specific activity[b] (Ci/mmol)	Yield[a] (%)	Radiochemical purity[c] (%)
0.1	15.0	54	3.4	150	23	94
0.15	12.0	63	4.8	80	40	98
0.3	9.0	63	3.6	30	40	93

[a]Yield in mCi and % yield relative to starting ^{32}Pi have not been corrected for radiochemical purity.

[b]Calculated from μmol H_3PO_4 and mCi ^{32}Pi added to monophosphate reaction mixture. No correction has been made for the 1.0 nmol unlabelled dATP added to the kinase reaction mixture for the synthesis of α-[^{32}P]dATP; this would be a maximum of 4%.

[c]Radiochemical purity was determined by thin-layer chromatography on PEI-cellulose in the presence of dAMP and dATP as markers and 0.5 M NH_4HCO_3 as solvent as described in the text.

(the dimethyl sulphoxide remains). A few drops of ethanol are added and evaporated to ensure complete removal of excess triethylamine.

3. ATP replaces dATP in the kinase reaction mixture for the conversion of [^{32}P]AMP to α-[^{32}P]ATP. The progress of the reaction is checked after 10 min and stopped after about 20 - 30 min.

The α-[^{32}P]ATP is finally dissolved in 1.5 ml 0.1 mM EDTA, pH 7, and stored at -15°. The yield and purity of the product are given in Table 2.

RESULTS AND DISCUSSION

A summary of three preparations of α-[^{32}P]dATP with specific activities from 30 - 150 Ci/mmole is given in Table 1. Yields varied from 23 - 40% relative to starting ^{32}Pi. The yields are lower than those obtained with α-[^{32}P]ATP (Table 2);

TABLE 2. Synthesis of α-[^{32}P]ATP

Carrier H_3PO_4 (μmol)	^{32}Pi (mCi)	2',3'-O-isopropylidene adenosine (μmol)	α-^{32}P ATP			
			mCi[a]	Specific activity[b] (Ci/mmol)	Yield[a] (%)	Radiochemical purity[c] (%)
0.1	13.0	53	7.3	130	56	99
0.15	12.0	50	8.2	80	68	99
0.3	6.3	46	4.2	21	66	99

[a]Yield in mCi and % yield relative to starting ^{32}Pi have not been corrected for radiochemical purity.

[b]Calculated from μmol H_3PO_4 and mCi ^{32}Pi added to monophosphate reaction mixture. No correction has been made for the 1.0 nmol unlabelled ATP added to the kinase reaction mixture for the synthesis of α-[^{32}P]ATP; this would be a maximum of 2%.

[c]Radiochemical purity was determined as in Table 1 but with markers of AMP, ADP and ATP.

this is due to the use of the unprotected 2'-deoxyadenosine in the chemical synthesis of the monophosphate in which both 5'-dAMP and 3'-dAMP are formed in a ratio of about 2:1 (2). Only the 5'-dAMP is then converted to the triphosphate by the myokinase-pyruvate kinase reaction.

The radiochemical purity as determined by thin-layer chromatography on PEI-cellulose varied from 93 - 98% although this would most likely be improved by further washing of the DEAE-cellulose column during the work-up procedure prior to elution of the α-[^{32}P]dATP; e.g. by washing with 25 ml of 0.07 M NH_4HCO_3 rather than 20 ml.

The α-[^{32}P]dATP prepared as described has been used extensively in this Department without dilution with unlabelled dATP for the reverse transcriptase catalysed synthesis of complementary DNA on RNA and for the preparation of labelled double-strand DNA by the 'nick translation' reaction catalysed by the Escherichia coli DNA polymerase I (6).

The results for three preparations of α-[^{32}P]ATP with specific activities of 20 - 130 Ci/mmol and yields of 56-68% are given in Table 2. A high radiochemical purity of 99% was obtained by the simple step-wise elution procedure from DEAE-cellulose. The α-[^{32}P]ATP has been used without dilution with unlabelled ATP for the terminal addition of poly(A) to the 3'-end of RNA by the E. coli poly(A) polymerase (7).

In the preparation of the monophosphate reaction mixture, it is important not to add more than about 1 µl of triethylamine and 1 µl of trichloroacetonitrile and not to incubate at 37° for longer than about 40 min. With higher levels of these reaction components and longer incubation times, the reaction mixture becomes dark yellow and an unknown inhibitory compound is formed in an amount which can drastically inhibit the subsequent kinase reaction. Fortunately, the addition of 1 µl of 2-mercaptoethanol to the kinase reaction mixture protects against this inhibitory effect under the reaction conditions described in Materials and Methods.

The fractionation of the triphosphate reaction mixture by step-wise elution from a small column of DEAE-cellulose was worked out with only one batch of DEAE-cellulose. It is

considered advisable to confirm that the procedure described also works with other batches of DEAE-cellulose.

Preparation of other α-[^{32}P]ribo- and deoxyribonucleoside 5'-triphosphates

Preliminary experiments have indicated that the methods described for the synthesis of α-[^{32}P]dATP and α-[^{32}P]ATP can be easily modified for the synthesis of other α-[^{32}P]triphosphates. For example, α-[^{32}P]dGTP was obtained in a yield of 60% relative to ^{32}Pi with a specific activity of 20 Ci/mmol and a radiochemical purity of 99%. For this synthesis, the myokinase in the reaction mixture was replaced by 50 µl of the partially purified nucleotide kinase preparation from E. coli (2,3) and the 1.0 nmol of either dATP or ATP was replaced by 2.8 µmol of ATP (final concentration 3.5 mM). The purification of the kinase reaction mixture by step-wise elution from a DEAE-cellulose column was the same except that the column was washed with 30 ml rather than 20 ml of 0.07 M NH_4HCO_3 prior to the elution of the α-[^{32}P]dGTP.

In the synthesis of α-[^{32}P]GTP by the same procedure, a minor problem is the insolubility of the guanosine (derived from the acid hydrolysis of the 2',3'-O-isopropylidene guanosine) on the addition of the nucleotide kinase reaction mixture. However, a homogeneous reaction mixture can be obtained by reducing the amount of isopropylidene guanosine used in the chemical phosphorylation step. Thus, starting with 0.2 µmol of ^{32}Pi and 15 µmol of isopropylidene guanosine, α-[^{32}P]GTP was obtained in a yield of 69% with a specific activity of 20 Ci/mmol and a radiochemical purity of 95%.

It should be remembered that, in using this modified procedure, the α-[^{32}P]triphosphates will be contaminated by essentially all of the unlabelled ATP used in the nucleotide kinase step.

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