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A method for the enzymatic synthesis and purification of [α-^{32}P] nucleoside triphosphates

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ABSTRACT

A simplified method is described for the enzymatic synthesis and purification of [α-^{32}P]ribo- and deoxyribonucleoside triphosphates. The products are obtained at >97% radiochemical purity with yields of 50-70% (relative to ^{32}Pi) by a two-step elution from DEAE-Sephadex. All reactions are done in one vessel as there is no need for intermediate product purifications. This method is therefore suitable for the synthesis of these radioactive compounds on a relatively large scale.

The sequential steps of the method involve first the synthesis of [γ-^{32}P]ATP and the subsequent phosphorylation of nucleoside 3' monophosphate with T_4 polynucleotide kinase to yield nucleoside 3',[5'-^{32}P]diphosphate. Hexokinase is used after the T_4 reaction to remove any remaining [γ-^{32}P]ATP. Nucleoside 3',[5'-^{32}P]diphosphate is treated with nuclease P-1 to produce the nucleoside [5'-^{32}P]monophosphate which is phosphorylated to the [α-^{32}P] nucleoside triphosphate with pyruvate kinase and nucleoside monophosphate kinase. Adenosine triphosphate used as the phosphate donor for [α-^{32}P] deoxynucleoside triphosphate syntheses is readily removed in a second purification step involving affinity chromatography on boronate-polyacrylamide. [α-^{32}P]Ribonucleoside triphosphates can be similarly purified when deoxyadenosine triphosphate is used as the phosphate donor.

INTRODUCTION

[α-^{32}P]Nucleoside triphosphates are used in many biochemical reactions. These can be synthesized by chemical[1] enzymatic[2,3] and combination chemical-enzymatic methods[4,5] All these approaches involve intermediate product purifications and/or manipulations which are inconvenient and potentially hazardous when dealing with large amounts of isotope. We describe here a simplified enzymatic synthesis which is done in one reaction vessel and involves only the sequential addition of substrates and enzymes.

MATERIALS AND METHODS

Carrier free [^{32}P] orthophosphoric acid in water was obtained from New England Nuclear at a concentration of 100 mCi/ml.

E. coli kinase was prepared according to the method of Hurlbert and Furlong.[6] Two ammonium sulfate fractions at 0.30-0.35 g/ml (Enzyme A) and 0.35-0.40 g/ml (Enzyme B) were obtained. Enzyme A was used for GMP phosphorylation. The activities of the individual nucleoside monophosphate kinases can be measured using the coupled pyruvate kinase-lactate dehydrogenase assay as described by Strominger et. al.[7]

T_4 polynucleotide kinase and beef liver nucleoside monophosphate kinase were obtained from P-L Biochemicals and Boehringer Mannheim, respectively. The nucleoside monophosphate kinase was dissolved in 50% glycerol to give 60 mg/ml and stored for up to two weeks.

The following enzymes were obtained from Sigma: Hexokinase type C-300; pyruvate kinase, Type II from rabbit muscle; nuclease P-1, dissolve 1 mg in 0.1 ml 1 M sodium acetate pH 5.0; glyceraldehyde-3-phosphate dehydrogenase as lyophilized and sulfate-free powder, dissolve 100 units in 0.5 ml water; 3-phosphoglyceric acid kinase as lyophilized and sulfate free powder, dissolve 1000 units in 0.5 ml deionized water.

All nucleotides were from Sigma.

Synthesis of [γ-^{32}P]ATP or [γ-^{32}P]dATP

All reactions are done in a thick wall screw capped test tube. The synthesis uses modifications of the method of Glynn and Chappell.[8] Combine the following: 100 µl deionized water, 50 µl 1 M Tris-HCl pH 8.0, 3.1 µl 1 M $MgCl_2$, 10 µl 5 mM EDTA, 50 µl 20 mM glutathione, 10 µl 50 mM sodium phosphoglycerate, 10 µl 50 mM NAD^+, 4 µl 25 mM ATP (or dATP). Add this cocktail to 250 µl (25 mCi) of ^{32}P, followed by 2 µl of glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate kinase (combine 1:1) and incubate 10 min at 37°. Longer incubations give reduced yields. At the completion of the reaction, boil for 3 min and chill on ice. Dilute approximately 1 µl into 1 ml of H_2O and spot 1 µl of this onto a PEI-cellulose thin-layer plate (Macherey-Nagel). The thin-layer plate is eluted with 0.5 M KH_2PO_4 (adjusted to pH 3.5 with concentrated HCl). Regions corresponding to [γ-^{32}P]ATP and ^{32}Pi are detected by autoradiography, cut from the plate, and counted by Cerenkov radiation to determine the yield of [γ-^{32}P]ATP.

Synthesis of Nucleoside 3',[5'-^{32}P]diphosphates

To the [γ-^{32}P]ATP reaction solution is added 10 µl 0.1 M spermine and 50 µl 25 mM 2' deoxynucleoside 3' monophosphate as one aliquot. Twenty-five units of T_4 polynucleotide kinase (2.5 µl) is added and the incubation is for 3 hrs. at 37°. Ribonucleoside 3' monophosphates are phosphorylated

using 10 units (1 µl) of T_4 polynucleotide kinase and [γ-^{32}P]dATP as the phosphate donor.

When the polynucleotide kinase reaction is complete, 1 µl hexokinase and 5 µl 1 M glucose are added in combination. An incubation for 10 min at 37° converts any remaining [γ-^{32}P]ATP to glucose-6-^{32}P.

Conversion of Nucleoside 3',[5'-^{32}P]diphosphates to Nucleoside [5'-^{32}P] monophosphates

Add 40 µl 1 M sodium acetate pH 5.0 to the previous reaction solution to give a final pH of 5.0-5.5. The ribonucleoside diphosphates are converted to ribonucleoside monophosphates by adding 1 µl of nuclease P-1 and incubating for 5 min at 37°. Longer incubation times (30 min for dADP, dGDP, dCDP, and 90 min for TDP) and more nuclease P-1 (5 µl) are required for the deoxynucleoside diphosphate cleavage. When the reaction is complete, the solution is boiled for 3 min to inactivate hexokinase.

Conversion of Nucleoside [5'-^{32}P]monophosphates to Nucleoside [α-^{32}P] triphosphates

Mix 125 µl 1 M Tris pH 8.0, 25 µl 0.1 M ATP, 5 mg phosphoenolpyruvate (potassium salt), and add to the previous reaction solution. The final pH should be approximately 7.5. Adenosine 5' triphosphate is used as a phosphate donor for the deoxynucleoside 5' monophosphates and vice-versa. Nucleoside monophosphate kinase and pyruvate kinase are added and the solution is incubated for the appropriate time at 37° (see Table 1). The solution is boiled for 3 min at the completion of the reaction.

Purification of [α-^{32}P] Nucleoside Triphosphates

1. [α-^{32}P]ribonucleoside triphosphates

The reaction solution is diluted to approximately 2 ml with deionized water and applied to a 2 ml DEAE-Sephadex (HCO_3^- form) column. Glucose-6-^{32}P and ^{32}Pi are eluted with 25 ml of 0.15 M ammonium bicarbonate pH 8.0. The nucleoside triphosphates are eluted with 10-15 ml of 0.5 M ammonium bicarbonate pH 8.0. These salt concentrations will probably vary with individual batches of DEAE-Sephadex.

Deoxyadenosine triphosphate is removed from the [α-^{32}P]ribonucleoside triphosphates by affinity chromatography on boronate-polyacrylamide (Affigel 601, Bio Rad Laboratories) as follows. Chill the 0.5 M ammonium bicarbonate fraction on ice and apply to a 6 ml column of Affigel 601 (packed in a disposable plastic syringe) which has been equilibrated with the same buffer. All operations are performed at 4°C. The column is eluted with 20 ml of chilled 0.5 M ammonium bicarbonate pH 8.0 to remove

TABLE 1. Enzyme Requirements for Conversion of Nucleoside [5'-^{32}P] *monophosphates to* [α-^{32}P]*nucleoside triphosphates.*

5' Nucleotide	Enzyme Mixture	Volume (μl)	Incubation Time
UMP	C	4	1 hr
CMP	C	4	1 hr
AMP	B	50	30 min
GMP	A	50	1 hr
TMP	B	100	5 hr
dCMP	B	100	4-5 hr
dAMP	B	100	30 min
dGMP	A	100	4-5 hr

Reaction solutions containing the appropriate nucleoside [5'-^{32}P] monophosphate were treated either with enzyme mixture A (E. coli kinase fraction A plus pyruvate kinase mixed 1:1), enzyme mixture B (E. coli kinase fraction B plus pyruvate kinase mixed 1:1) or enzyme mixture C (beef liver nucleoside monophosphate kinase plus pyruvate kinase mixed 1:1). Enzyme mixtures A and B were dialysed at 4° against 50 mM Tris pH 7.6, 30 mM $MgCl_2$, 2 mM KCl for 6 hrs before use. These dialysed preparations are stable for at least 1 month.

dATP and any remaining traces of ^{32}Pi. Elution is continued with 0.25 M ammonium formate pH 4.5 to displace the [α-^{32}P]nucleoside triphosphate. The product is eluted in 12-15 ml, several drops of ammonium hydroxide are added to bring the pH to near neutrality, and the ^{32}P-nucleotide is stored at -20°C.

2. [α-^{32}P]deoxynucleoside triphosphates

The procedure is similar to that used for the ribonucleoside purification except that the DEAE-Sephadex (acetate form) column is eluted sequentially with 0.15 M ammonium acetate pH 8.0 and 0.5 M ammonium acetate pH 8.0. The 0.5 M ammonium acetate fraction is applied to a 6 ml column of Affigel 601 which has been equilibrated with 0.5 M ammonium acetate pH 8.0. Adenosine 5' triphosphate is retained by the affinity column while the ^{32}P-deoxy-nucleotide is eluted in a volume of 6-10 ml.

Electrophoresis and Chromatography

Aliquots (1 μl) from the reaction were analyzed by electrophoresis on Whatman 1 MM (55 cm strips) for 20 minutes at 55 V/cm using 0.5% pyridine, 5% acetic acid, 5 mM EDTA. Samples were alternatively chromatographed on polyethyleneimine-cellulose thin-layers (Macherey-Nagel) using 0.5 M

KH_2PO_4 adjusted to pH 3.5 with HCl.

RESULTS AND DISCUSSION

A method has been established for the enzymatic synthesis of [α-^{32}P] nucleoside triphosphates which does not require the purification of intermediate reaction products. This procedure utilizes the T_4 polynucleotide kinase catalyzed exchange between [γ-^{32}P]ATP and nucleoside 3' monophosphates[9], followed by hexokinase treatment to destroy any remaining [γ-^{32}P]ATP. The nucleoside 3',[5'-^{32}P]diphosphates are converted by nuclease P-1 to the [5-^{32}P]mononucleotide and then phosphorylated to the [α-^{32}P]nucleoside triphosphate using nucleoside monophosphate kinase and pyruvate kinase.

Approaches similar to this have been used for the synthesis of [α-^{32}P] nucleoside triphosphates; however, these used intermediate purifications of [γ-^{32}P]ATP and the nucleoside [5'-^{32}P]monophosphates[2,3]. We find it unnecessary to purify [γ-^{32}P]ATP before the T_4 kinase step. Furthermore, since the conversion of nucleoside [5'-^{32}P]monophosphate to [α-^{32}P]nucleoside triphosphate requires ATP, the destruction of [γ-^{32}P]ATP after the T_4 kinase reaction eliminates the need to isolate the nucleoside [5'-^{32}P]monophosphate intermediate.

The reaction steps are most easily monitored by electrophoresis on Whatman 1 MM paper using pyridine-acetate pH 3.5. Examples of the individual reactions for the synthesis of the four [α-^{32}P]ribonucleoside triphosphates are shown in Figure 1. The conversion of the eight common nucleoside [5'-^{32}P] monophosphates to the corresponding [α-^{32}P]nucleoside triphosphates is shown in Figure 2.

Purification of the [α-^{32}P] Nucleoside Triphosphates

The major contaminants after the [α-^{32}P]nucleoside triphosphate synthesis are glucose-6-^{32}P,^{32}Pi, ATP (or dATP), and the corresponding nucleoside (from the 3'NMP→nucleoside conversion catalyzed by nuclease P-1). Chromatography of the reaction solution using a two-step elution from DEAE-Sephadex yields gives products free from the parent nucleoside and with >97% radiochemical purity (see Materials and Methods). Adenosine 5' triphosphate may be removed from the product at this stage using conventional methods such as ion-exchange or electrophoresis; however, these approaches are neither convenient nor safe when handling large amounts of isotope.

We, therefore, developed the following procedure as a simple alternative for removing ATP. [α-^{32}P]ribonucleoside triphosphates are synthesized using dATP as the phosphate donor and ATP is used as the donor for [α-^{32}P]deoxy-

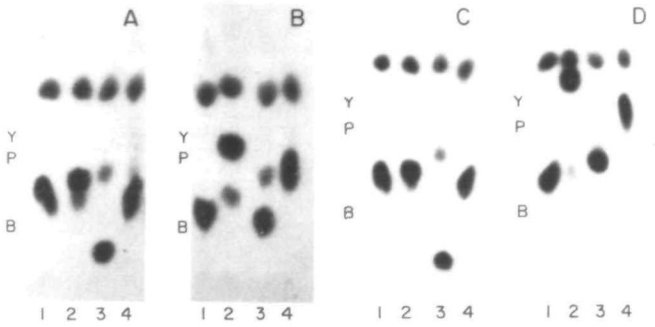

Figure 1. Electrophoretic separation of intermediate reaction products in the synthesis of [α-^{32}P]ribonucleoside triphosphates.

[α-^{32}P]ribonucleoside triphosphates were synthesized as described in Materials and Methods. Aliquots (1 µl) were withdrawn after each reaction step and spotted on 55 cm strips of Whatman 1 MM. Electrophoresis was done at 55 volts/cm for 20 min using a pyridine-acetate-EDTA pH 3.5 buffer.

The synthesis of [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP and [α-^{32}P]UTP correspond to A, B, C, and D, respectively. Individual steps in the synthesis are: [γ-^{32}P]dATP and ^{32}Pi from [γ-^{32}P]dATP synthesis (lane 1); ribonucleoside 3',[5'-^{32}P]diphosphate and residual [γ-^{32}P]dATP from the T_4 polynucleotide kinase reaction (lane 2); nucleoside [5'-^{32}P]monophosphate and glucose-6-^{32}P from nuclease P-1 and Hexokinase steps (lane 3); [α-^{32}P] nucleoside triphosphate from the nucleoside monophosphate kinase and pyruvate kinase reactions (lane 4).

Marker dyes used were acid fuchsin (P), xylene cyanol (B), and orange G (Y).

nucleoside triphosphate syntheses. After the initial purification of the ^{32}P-nucleotide product on DEAE-Sephadex, the product is chromatographed on boronate-acrylamide (Affigel 601) to separate the donor nucleotide from the [α-^{32}P]nucleoside triphosphate. This approach takes advantage of the ability of ribonucleotides to bind through their 2',3' cis diol groups to a boronate affinity matrix[10] The fractionation of a [^{3}H]dATP/[γ-^{32}P]ATP mixture on Affigel 601 is shown in Figure 3. Adenosine triphosphate is retained by the affinity column at pH 8 and displaced by a low pH buffer, while dATP is eluted quantitatively at the higher pH. A small fraction of the ^{32}P-radioactivity (3%) not bound to the affinity column corresponds to a ^{32}Pi contaminant in the [γ-^{32}P]ATP preparation used for this experiment. Affigel 601 is a polyacrylamide matrix which has a high capacity, good swelling properties, and gives excellent separations provided that elutions are done at 4° and the column volume is not less than 5 ml. Although

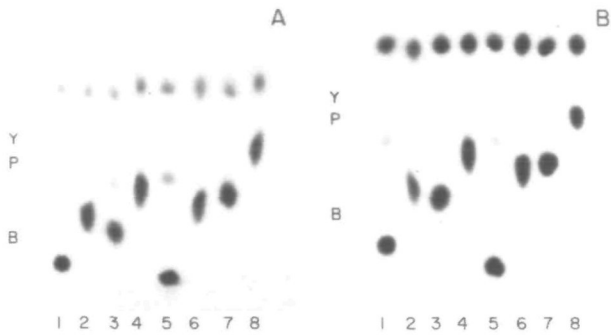

Figure 2. Conversion of nucleoside [5'-^{32}P]monophosphates to [α-^{32}P] nucleoside triphosphates.

Nucleoside [5'-^{32}P]monophosphates were synthesized and converted to the [α-^{32}P]nucleoside triphosphates as described in Materials and Methods. Aliquots were electrophoresed on Whatman 1 MM at pH 3.5 (see Materials and Methods).

Panels A and B correspond to the ribonucleotides and deoxynucleotides respectively. The numbered lanes in panel A correspond to: AMP and ATP (lanes 1 and 2), GMP and GTP (lanes 3 and 4), CMP and CTP (lanes 5 and 6), UMP and UTP (lanes 7 and 8). The numbered lanes in panel B correspond to the deoxynucleotide counterparts of panel A. The fastest moving compound in all lanes is ^{32}Pi. Glucose-6-^{32}P seen in lanes 1, 3, 5, and 7 moves slightly behind the acid fuchsin dye (P). Marker dyes were the same as in Figure 1.

dihydroxyboryl-cellulose has been used for the separation of deoxyribonucleoside monophosphates from ribonucleoside monophosphates;[10] we found that commercial preparations give poor separations of the nucleoside triphosphates.

Choosing the complementary deoxy or ribonucleotide as a phosphate donor has several advantages apart from simplifying the product purification. Synthesis of [α-^{32}P]ATP and [α-^{32}P]dATP at high specific activity can be achieved since the labelled nucleotide is not diluted with the donor nucleotide. Furthermore, in some cases the affinity chromatography separation might not even be necessary since the complementary nucleotide phosphate donor will not interfere with subsequent enzyme reactions, e.g. RNA and DNA polymerases.

Factors Influencing the [α-^{32}P]Nucleoside Triphosphate Yield

The most important factor influencing the [α-^{32}P]nucleoside triphosphate yield is the yield of [γ-^{32}P]ATP formed in the first step of the synthesis.

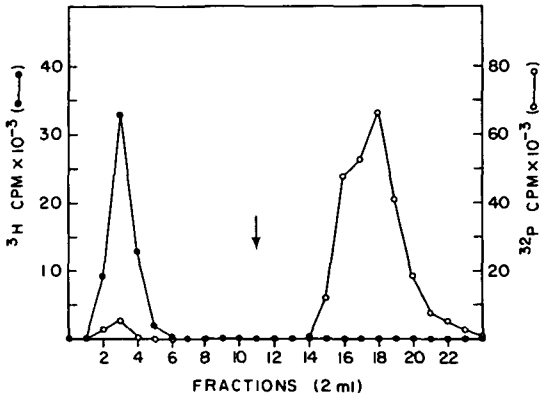

Figure 3. Separation of ATP and dATP by Chromatography on Affigel 601. [γ-^{32}P]ATP (3.8 nmole) and [^{3}H]dATP (3.8 nmole) were applied in 0.5 M ammonium bicarbonate pH 8 to a 6 ml column of Affigel 601 equilibrated with the same buffer. The column was eluted at 4° first with 0.5 M ammonium bicarbonate pH 8 for 10 fractions (2 ml) to remove [^{3}H]dATP and then with 0.25 M ammonium formate pH 4.5 to displace [γ-^{32}P]ATP.

As shown in Table 2, the yields of both [γ-^{32}P]ATP and [γ-^{32}P]dATP increase with increasing ATP (or dATP) concentrations. Our reactions use nucleotide concentrations (100 nmoles/500 µl reaction volume) which routinely give [γ-^{32}P]nucleotide yields of 75-85%. Higher yields are possible by increas-

TABLE 2: The Effect of ATP and dATP Concentration on [γ-^{32}P]Nucleoside Triphosphate Yield.

nmole ATP or dATP in reaction	% [γ-^{32}P]ATP	% [γ-^{32}P]dATP
250	86	81
100	78	75
25	50	49
12.5	34	36
5	12	16

[γ-^{32}P]Nucleoside triphosphate syntheses were done in a 0.5 ml reaction volume containing 2.7 nmoles K_2HPO_4 and 250 µCi ^{32}Pi (see Materials and Methods). Reactions were for 10 minutes at 37° and aliquots were analyzed by chromatography on PEI-cellulose using 0.5 M KH_2PO_4 pH 3.5 (adjusted with HCl) as the solvent. Regions corresponding to the [γ-^{32}P] nucleotide and ^{32}Pi were detected by autoradiography, cut from the plate, and radioactivity was determined using Cerenkov radiation.

ing the ATP concentration further; however, as discussed below, this leads to a reduction in the specific activity of the [α-^{32}P]nucleoside triphosphate product.

Final yields of the [α-^{32}P]nucleoside triphosphate vary from 50-70% relative to the input ^{32}Pi. Yields approaching that of [γ-^{32}P]ATP can be obtained by increasing the yield of the T_4 kinase step with either longer incubations or more enzyme. As shown by others,[11] spermine was essential to obtain high levels of phosphate transfer. It is advisable to check the entire synthesis on a small scale since different enzyme preparations will vary in activity. Most reactions in this study were done in a volume of 500 µl containing 2.7 nmole of inorganic phosphate and 250 µCi of ^{32}Pi which corresponds to a total phosphate concentration equivalent to that of 25 mCi of ^{32}Pi in 500 µl. Reduction of the ^{32}Pi specific activity does not affect the ^{32}P-nucleotide yield provided that the phosphate concentration is not changed and large syntheses (25 mCi) give similar yields.

Determination of the Specific Activity of [α-^{32}P]Nucleoside Triphosphates

The specific activity of the [α-^{32}P]nucleotide product is equal to the specific activity of [γ-^{32}P]ATP (or [γ-^{32}P]dATP) formed in the first reaction. Three factors determine the [γ-^{32}P]ATP specific activity: the concentration of ATP in the synthesis, the yield of [γ-^{32}P]ATP, and the amount of isotope used (which is kept constant at 25 mCi). As shown in Table 2, the first two of these parameters are interrelated, that is, as the ATP concentration is decreased the [γ-^{32}P]ATP yield also decreases. Therefore, although it is possible to synthesize [α-^{32}P]nucleoside triphosphates at very high specific activities, the yields will be correspondingly lower. We use ATP concentrations which give [γ-^{32}P]ATP with a yield of 75-85% and a specific activity of about 200 Ci/mmole. This specific activity is suitable for most purposes.

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