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A Simple Enzymic Method for the Synthesis of Adenosine 5′-[α-^{32}P]Triphosphate on a Preparative Scale

By B. RICHARD MARTIN and H. PAUL VOORHEIS
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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A simple, rapid and inexpensive method is described for the enzymic synthesis of [α-^{32}P]ATP from [^{32}P]P_i on a preparative scale with an overall yield of 53%. The final product contained all of the detectable radioactivity (>99.9%) in the α position and has been shown to behave identically with commercially available [α-^{32}P]ATP during the synthesis of 3′:5′-cyclic AMP in the reaction catalysed by adenylate cyclase.

A major use for [α-^{32}P]ATP is found in the assay of adenylate cyclase. Since the activity of this enzyme in all cells is relatively low, large amounts of labelled ATP are required to achieve a sufficient substrate specific radioactivity for accurate measurements. Therefore any major investigation is extremely expensive, owing to the high cost of commercially available [α-^{32}P]ATP; it seemed desirable to develop a method for the synthesis of [α-^{32}P]ATP from [^{32}P]P_i which would be comparatively inexpensive, rapid, safe and convenient.

The method commonly used at present involves at one stage the chemical synthesis of [^{32}P]AMP (Symons, 1968) at a temperature of 95°C and requires a vacuum line and five evaporations to dryness under reduced pressure. This procedure could be potentially dangerous in the average biochemical laboratory. The yield is about 50% and requires considerable time and many manipulations.

An enzymic procedure with a high yield of [α-^{32}P]ATP has the advantages that all the reactions can be performed in aqueous solution at 37°C or below in open vessels and that the amount of starting radioactivity can be kept to a minimum. Thus specialized equipment is not required and the hazard from radiation can be dealt with by normal laboratory practice. The method described below can be completed in 1 day and gives a yield equivalent to 50–70% of the original [^{32}P]P_i used with all of the radioactivity in the α-position.

The procedure can be divided into three stages: (I) synthesis of [γ-^{32}P]ATP by a modification of the method of Glynn & Chappell (1964); (II) production of [^{32}P]AMP by the reaction catalysed by adenosine kinase (ATP–adenosine 5′-phosphotransferase, EC 2.7.1.20):

$$\text{Adenosine} + \text{ATP} \rightarrow \text{AMP} + \text{ADP}$$

(III) production of [α-^{32}P]ATP from [^{32}P]AMP by the reactions catalysed by adenylate kinase (ATP–AMP phosphotransferase, EC 2.7.4.3) and creatine phosphokinase (ATP–creatine phosphotransferase, EC 2.7.3.2):

$$\text{AMP} + \text{ATP} \rightarrow 2\text{ADP}$$
$$\text{ADP} + \text{creatine phosphate} \rightarrow \text{ATP} + \text{creatine}$$

Materials and Methods

ATP, ADP, AMP, adenylate kinase, glyceraldehyde 3-phosphate dehydrogenase, acetyl-CoA synthetase, inorganic pyrophosphatase, phosphoglycerate kinase and 3-phosphoglycerate (trisodium salt) were obtained from Boehringer (London) Ltd., Bell Lane, Lewes, East Sussex BN7 1LG, U.K. Creatine phosphate and creatine phosphokinase were obtained from Sigma (London) Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, U.K. All other reagents were of analytical grade.

Assay of adenylate kinase

For all enzyme assays 1 unit of activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol of substrate into product in 1 min. Adenylate kinase was assayed by the method of Oliver (1955) in the direction of ATP production by coupling to the hexokinase and glucose 6-phosphate dehydrogenase reactions.

Assay of adenosine kinase

Adenosine kinase was assayed by the rapid method of Murray (1968), with [U-^{14}C]adenosine and ATP as substrates. Unchanged [U-^{14}C]adenosine was removed from the reaction mixture (2ml) by six successive extractions with a solution (5ml) of butan-1-ol saturated with water. The product [U-^{14}C]AMP was measured by liquid-scintillation counting. The scintillation mixture for radioactive counting contained 10g of butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] and 500g of Triton X-100 made up to 2.5 litres with toluene.

Preparation of adenosine kinase

The enzyme was prepared from *Saccharomyces cerevisiae* by a modification of the method of Kornberg & Pricer (1951). All procedures were carried out at 4°C unless otherwise stated. Fresh baker's yeast (0.45kg; 1lb) was homogenized by grinding with Ballotini beads in a motor-driven pestle and mortar. The resulting paste was extracted with 3×400ml of 50mM-potassium phosphate buffer (pH6.8) containing NaN_3 (0.5mM) and adenosine (2mM). The extracts were pooled (total volume approx. 1.2 litres) and then centrifuged at 22000*g* for 10min. Potassium phosphate (0.5M) at pH7.4 was added to the decanted cloudy supernatant (100ml/litre of supernatant) to give a final concentration of 0.1M-P_i. $CaCl_2$ (0.5M) was then added rapidly with stirring to give a final Ca^{2+} concentration of 0.05M. The suspension was left for 30min and then centrifuged for 10min at 22000*g*. The clear supernatant was decanted and acidified to pH4.4 by the gradual addition of 1M-acetic acid (glass electrode). The suspension was centrifuged at 22000*g* for 10min. The supernatant was decanted and adjusted to pH5.1 by the addition of 1M-NaOH. Ethanol (150ml/litre of supernatant) was added over a period of 30min with stirring. During this addition the temperature was gradually decreased from +5°C to −1°C by using an acetone/solid CO_2 freezing bath. Then 60ml of 0.5M-$ZnCl_2$ was added with stirring for each litre of the original solution. The suspension was centrifuged at 22000*g* for 10min. The supernatant was decanted and 580ml of ethanol/litre of supernatant was added over a period of 30min with stirring, during which time the temperature was decreased to −5°C. The suspension was centrifuged at 22000*g* for 20min. The pellet was redissolved in 50mM-potassium phosphate buffer (pH7.5) and freeze-dried. The procedure yielded 6g of a dry white powder containing 1.0g of protein, 700 units of adenosine kinase. The preparation was heavily contaminated with adenylate kinase, but there was almost no detectable adenosine triphosphatase activity (Fig. 1).

Assay of adenylate cyclase

Adenylate cyclase was assayed by the method of Salomon *et al.* (1974). Liver plasma membranes prepared by the method of Pilkis *et al.* (1974) were used as the source of the enzyme.

Analytical nucleotide separation and assay for content of ^{32}P

Separation of adenine nucleotides was accomplished on polyethyleneimine-impregnated cellulose-coated thin-layer plastic sheets (Machery Nagel and Co., 516 Düren Postfach 307, Germany) by the method of Randerath & Randerath (1967), with 2M-formic acid/sodium formate (pH3.4) as solvent. The identity of the various components was checked in several experiments by developing parallel chromatograms in a second solvent of 1M-LiCl simply to confirm the routine results obtained with the first solvent system. The sheets were prewashed by running three times in 1M-LiCl before use in either solvent system. Salt was removed from the layers by rinsing in methanol and then drying before use.

Radioactivity (^{32}P) in strips cut from the chromatogram was detected by Čerenkov counting in water by using a liquid-scintillation counter.

Synthesis of [α-^{32}P]ATP

(*I*) *Preparation of [γ-^{32}P]ATP.* The procedure of Glynn & Chappell (1964) was followed. The incubation mixture contained 100mM-Tris/HCl, pH8.0, dithiothreitol (1mM), $MgCl_2$ (6mM), ATP (3.4mM), 3-phosphoglyceric acid (0.5mM), NAD^+ (0.1mM), glyceraldehyde 3-phosphate dehydrogenase (0.02mg), 3-phosphoglycerate kinase (0.1mg) and 10–20mCi of [^{32}P]P_i in 0.1M-HCl, all in a total volume of 3ml. The mixture was incubated at 30°C for 30min and the procedure terminated by placing the reaction tube in a boiling-water bath for 3min. Denatured protein was removed by centrifugation (1000*g*, 5min).

Glynn & Chappell (1964) found that the NAD^+ in the preparation of glyceraldehyde 3-phosphate dehydrogenase was sufficient to support the exchange of [^{32}P]P_i with ATP, giving yields of [γ-^{32}P]ATP in excess of 90%. However, with the preparation we used, the incorporation of ^{32}P into ATP was only about 10% in the absence of added NAD^+, but in the presence of NAD^+ yields were in excess of 90%. This difference presumably reflects differences in the nucleotide content of the commercially available enzyme.

(*II*) *Preparation of [^{32}P]AMP.* The supernatant from the first stage was mixed with 3ml of 25mM-adenosine and 250mg of the adenosine kinase preparation giving a total volume of approx. 6ml. The mixture was incubated for 2½h at 37°C and the reaction terminated by the addition of an equal volume of ethanol. The precipitated protein was removed by centrifugation (1000*g* for 5min); the supernatant was diluted to 200ml with water and applied to a column (2ml bed volume; 2cm high×1cm diam.; 20°C) of Dowex 1 (Cl^- form) which had been thoroughly washed with distilled water. After application of the sample the column was washed with 50ml of water to remove salts and adenosine, then by 10ml of 0.01M-HCl which was discarded. The AMP was eluted with a further 50ml of 0.01M-HCl which contained the bulk of the [^{32}P]AMP. This fraction was neutralized (pH7.4) with 1M-Tris (free base) and used for the last stage of the procedure.

(III) Preparation of [α-^{32}P]ATP. The product from the second stage was mixed with 0.25ml of 1M-$MgCl_2$, 0.25ml of 100mM-ATP, creatine phosphokinase (600 units), adenylate kinase (36 units) and creatine phosphate (220μmol) and then incubated at 37°C (pH7.4) for 1h. The reaction was stopped by the addition of 50ml of ethanol and the precipitated protein removed by centrifugation (1000*g* for 5min). The supernatant was applied to a column (1ml bed volume, 1cm high×1cm diam.; 20°C) of Dowex 1 (Cl^- form). The column was then washed with 100ml of 0.04M-HCl to remove [^{32}P]P_i, [^{32}P]AMP and [^{32}P]ADP. The [α-^{32}P]ATP was eluted from the column with 10ml of 0.25M-HCl, neutralized (pH7.0) with 2.5ml of 1M-Tris (free base) and immediately frozen in batches of 0.5ml which were stored at −20°C until used. The ATP concentration in the final solution was approx. 1mM.

Results and Discussion

Fig. 1 shows the time-course of production of [^{32}P]AMP from [γ-^{32}P]ATP. At the start of the incubation 98% of the original [^{32}P]P_i was in ATP and 2% in P_i. During the incubation the amount of ^{32}P in ADP rose rapidly to a maximum of 42% and then gradually fell to a value of 25%. ^{32}P in AMP rose steadily to a maximum of 67%, whereas ^{32}P in P_i showed a very gradual rise to a value of 5%. Another 3% remained in ATP. The incorporation of label into ADP resulted from the presence of adenylate kinase in the adenosine kinase preparation. Since the equilibrium of the adenosine kinase reaction is markedly in favour of AMP production whereas the equilibrium constant of the adenylate kinase reaction is close to unity it should have been possible to convert all the ADP into AMP either directly or via ATP. However, after 3h the rate of increase of ^{32}P incorporation into AMP became very low, presumably because the ATP concentration was well below the K_m value for adenosine kinase by this time. For convenience the process was usually terminated at this stage. If the reaction had been allowed to go to completion all of the phosphate of ATP would have been incorporated into AMP.

The overall reaction would then be:

$$\text{ATP} + 2\ \text{adenosine} \rightarrow 3\ \text{AMP}$$

Thus the specific radioactivity of the AMP should have approached one-third of the value of the original [γ-^{32}P]ATP. In fact, the specific radioactivity was about half of this value (see Table 1) when the reaction was stopped.

Recovery of ^{32}P

Table 1 shows the results of a typical preparation. The recovery of ^{32}P in the terminal phosphate of ATP

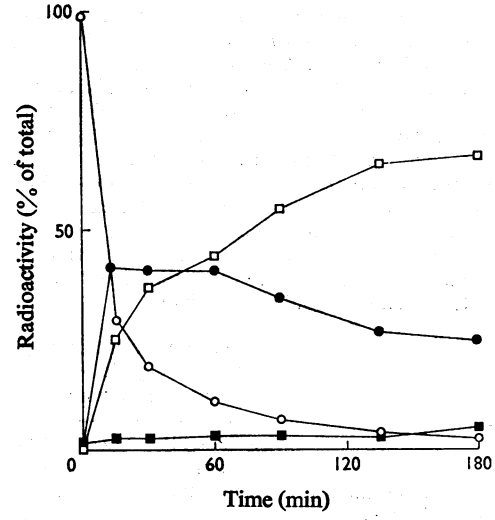

Fig. 1. *Time-course of the incorporation of ^{32}P derived from [γ-^{32}P]ATP into AMP (□), ADP (●), ATP (○) and P_i (■)*

For details of incubation see the Materials and Methods section. Samples were withdrawn at timed intervals and subjected to chromatography on polyethyleneimine–cellulose thin-layer sheets. The chromatograms were cut into 1cm strips and their radioactivity was determined. The position of each nucleotide and P_i was determined by using appropriate standards.

Table 1. *Yield (%) and specific radioactivity of ^{32}P in intermediates and the product of the enzymic synthesis of [α-^{32}P]ATP*

See the introduction and Materials and Methods section for details of the synthetic steps. Samples were taken at various stages in the synthesis and the A_{260} was measured in a Unicam SP.500 spectrophotometer. After chromatography on polyethyleneimine–cellulose thin-layer sheets the adenine nucleotide spots were cut out and their radioactivity was measured.

Stage of synthesis	Product	Yield of ^{32}P (%)	Specific radioactivity (mCi/mmol)
Stage (I)	[γ-^{32}P]ATP	92	1200
Stage (II)	[^{32}P]AMP+ other products	70	—
Purification of AMP on Dowex 1	[^{32}P]AMP	64	690
Stage (III)	[α-^{32}P]ATP+ other products	59	—
Purification of ATP on Dowex 1	[α-^{32}P]ATP	53	645

Table 2. *A comparison of [α-^{32}P]ATP commercially available and that produced enzymically as a substrate for adenylate cyclase from rat liver membranes*

Liver plasma membranes (30 μg/assay) were incubated at 30°C for 15 min in a total volume of 100 μl containing Tris/HCl (25 mM) at pH 7.4, $MgCl_2$ (10 mM), 3′:5′-cyclic AMP (0.1 mM), creatine phosphate (10 mM), creatine phosphokinase (50 units/ml), ATP (0.5 mM) and [α-^{32}P]ATP (1 μCi) obtained commercially (The Radiochemical Centre) or prepared enzymically as described in the text. Each value is the mean ± S.E.M. of three determinations. GMP-P(NH)P, guanylyl imidodiphosphate.

Additions to standard assay mixture (μM)	Adenylate cyclase activity (pmol of cyclic AMP produced/15 min)	
	[α-^{32}P]ATP from commercial source	[α-^{32}P]ATP from enzymic synthesis
None	15.7 ± 0.83	14.5 ± 0.27
NaF (10^4)	91.0 ± 2.7	91.7 ± 0.52
GMP-P(NH)P (10)	50.3 ± 0.92	45.6 ± 2.3
Glucagon (1)	52.4 ± 1.6	57.8 ± 1.3
GMP-P(NH)P (10) + glucagon (1)	104.0 ± 3.0	102.0 ± 4.5

at the end of the first stage of the procedure was in excess of 90%. Starting with 20 mCi of [^{32}P]P_i and 15 μmol of unlabelled ATP, a specific radioactivity of [γ-^{32}P]ATP of about 1200 mCi/mol resulted. This value will limit the specific radioactivity of the final product and can be increased, either by increasing the amount of [^{32}P]P_i used, or decreasing the amount of unlabelled ATP.

About 70% of the original [^{32}P]P_i was incorporated into AMP at the end of the second stage of the procedure in the reaction with adenosine kinase; the bulk of the remainder was in ADP. After purification on Dowex 1 the amount of original [^{32}P]P_i in AMP (690 mCi/mmol) was decreased to 64%.

At the last stage of the synthesis 59% of the original [^{32}P]P_i was incorporated into [α-^{32}P]ATP (645 mCi/mmol) and this yield was decreased to 53% after purification on Dowex 1. The specific radioactivity of the final [α-^{32}P]ATP was decreased slightly from that of the [^{32}P]AMP as a result of the addition of unlabelled ATP to initiate the final reaction.

Purity of [^{32}P]AMP and [α-^{32}P]ATP

After purification on Dowex 1, the intermediate, [^{32}P]AMP, contained no detectable [^{32}P]ADP or [^{32}P]ATP. However, it was contaminated to the extent of about 6% by [^{32}P]P_i. P_i is not a substrate for either adenylate kinase or creatine kinase so that it cannot be incorporated into ATP. Therefore the only source of ^{32}P in ATP was [^{32}P]AMP and the label must be incorporated into the α-position of the ATP.

The purified [α-^{32}P]ATP contained less than 0.2% of label in components other than ATP. As a further check on the quality of the product the [α-^{32}P]ATP prepared by this method was compared with [α-^{32}P]ATP purchased from The Radiochemical Centre, Amersham, Bucks., U.K., in the assay of adenylate cyclase. This enzymic reaction is particularly suited for an investigation of the quality of the ATP synthesized by a new method, since there are reported to be a number of different intermediate states of the enzyme, generated by different effectors, each binding ATP and catalysing the formation of cyclic AMP at different rates (Rodbell *et al.*, 1975). Rat liver plasma membranes containing adenylate cyclase were assayed for basal activity and activity in the presence of the activators, NaF, guanylyl imidodiphosphate and glucagon. Table 2 shows that the values obtained with [α-^{32}P]ATP prepared as described in the method reported here and with commercial [α-^{32}P]ATP are in excellent agreement.

Positional analysis of the ^{32}P labelling of the final product

The location of ^{32}P within the ATP molecule was examined by making use of the reactions catalysed by acetyl-CoA synthetase [acetate–CoA ligase (AMP-forming), EC 6.2.1.1] and inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1).

$$\text{Acetate} + \text{ATP} + \text{CoASH} \rightleftharpoons \text{acetyl-CoA} + \text{AMP} + \text{P–P}_i$$

$$\text{P–P}_i \rightarrow 2\text{P}_i$$

The reaction mixture (1 ml final volume) contained 50 mM-Tris/HCl (pH 7.4), ATP (4 mM), 5 μCi of [α-^{32}P]ATP prepared by the enzymic procedure, $MgCl_2$ (5 mM), CoASH (6 mM), sodium acetate (10 mM), bovine serum albumin (0.1%), 2-mercaptoethanol (1 mM), acetyl-CoA synthetase (1 unit) and inorganic pyrophosphatase (1 unit).

The reaction mixture was incubated until only 22% of the ^{32}P remained in ATP (10 min, 30°C) and was stopped by immersing the tube in a boiling-water bath for 3 min. All of the ^{32}P removed from ATP (78%) was in AMP. No radioactivity was detected in P_i. The limit of detection was 0.1% of the total radioactivity. Therefore at least 99.9% of the radioactivity in the ATP that was degraded by this analytical technique was in the α-phosphate.

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