A Novel Method for the Synthesis of Nucleoside Triphosphates Labelled with Inorganic [^{32}P]Phosphate Specifically in the β -Position

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A method was developed for the introduction of [^{32}P]P_i specifically into the β -position of ATP and GTP. The method is based on two separate reactions involving (a) phosphorolysis of poly(A) or poly(G) [Soreq, Nudel, Salomon, Revel & Littauer (1974) *J. Mol. Biol.* **88**, 233–245] in the presence of [^{32}P]P_i and (b) conversion of the labelled diphosphate into the corresponding triphosphate by transferring the active phosphate group from 1,3-diphosphoglycerate in a coupled reaction as described by Glynn & Chappell [(1964) *Biochem. J.* **90**, 147–149]. Radioactivity in the β - and γ -phosphate groups of the labelled triphosphate was measured by using polynucleotide kinase. No detectable radioactivity was found in the γ -phosphate group. The suitability of this method for the synthesis of other nucleoside triphosphates specifically labelled in the β -position is discussed.

Nucleoside triphosphates labelled specifically in the β -position with [^{32}P]P_i are not available commercially. Although triphosphates labelled in both β - and γ -positions can be obtained, such compounds were found unsuitable in certain experiments designed to study the origin of the middle phosphate group of cap structures in eukaryotic mRNA.

The present paper describes a novel method for the synthesis of nucleoside triphosphates labelled exclusively in the β -position. The method is based on two separate reactions involving (a) phosphorolysis of polynucleotides, e.g. poly(A), in the presence of [^{32}P]P_i, and (b) conversion of the labelled diphosphate into triphosphate, by making use of a coupled reaction using glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase.

Surrey, U.K.). 3-Phosphoglycerate kinase (EC 2.7.2.3) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) were supplied as a mixture of activity ratio 1:1 by Boehringer Mannheim G.m.b.H. (Mannheim, W. Germany). Carrier-free [^{32}P]P_i was from The Radiochemical Centre (Amersham, Bucks., U.K.). Polynucleotide kinase (EC 2.7.1.78) was a gift from Mr. J. R. Lillehaug (University of Bergen).

Enzymic reactions

Phosphorolysis of poly(A) and poly(G). The phosphorolysis reaction was carried out essentially as described in the literature (Kaufmann *et al.*, 1973; Soreq *et al.*, 1974). The reaction mixture contained, in a final volume of 500 μl : 5 mCi of carrier-free [^{32}P]P_i (freeze-dried after neutralization with NH₃), 2 mM-K₂HPO₄, 100 mM-Tris/HCl (pH 8.2), 1 mM-EDTA, 0.5 μmol of P of poly(A) or poly(G), 10 mM-MgCl₂ and 20 μg of polynucleotide phosphorylase (50 units/mg of protein). The mixture was incubated at 25°C for 2 h. At the end of the incubation period the mixture was diluted with 4 ml of 20 mM-tris-(hydroxyethyl)ammonium acetate buffer (pH 5.0).

Conversion of nucleoside diphosphate into triphosphate. Radioactive nucleoside diphosphate eluted from a DEAE-Sephadex column was freeze-dried and converted into triphosphate by transferring the active phosphate group from 1,3-diphosphoglycerate in a coupled reaction as described by Glynn & Chappell (1964). Unlabelled P_i and another nucleoside triphosphate were used to regenerate 1,3-diphosphoglycerate.

Experimental

Materials

Poly(A) (potassium salt) was from Miles Laboratories (Elkhart, IN, U.S.A.). Polynucleotide phosphorylase (EC 2.7.7.8), ATP, GTP and 3-phosphoglycerate (sodium salt) were from Sigma (London) Chemical Co. (Kingston-on-Thames KT2 7BH,

The reaction mixture contained, in a final volume of 1 ml: 0.1 mM labelled ADP or GDP, 0.5 mM unlabelled GTP or ATP, 3 mM-reduced glutathione, 50 mM-Tris/HCl (pH 7.5), 10 mM-MgCl₂, 0.3 mM-3-phosphoglycerate, 1 mM-P_i and 10 units each of 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase. After a 45 min incubation at 25°C, the mixture was diluted with 4 ml of water.

Separation and purification of labelled nucleoside diphosphate and triphosphate

³²P-labelled nucleoside diphosphate produced by the phosphorolysis of poly(A) or poly(G) was purified by chromatography on DEAE-cellulose by using a 200 ml gradient of 0.02–1 M-tris(hydroxyethyl)-ammonium acetate buffer (pH 5.0) as described by Nomoto *et al.* (1976). ADP and GDP were eluted from the column by 0.3 M and 0.36 M buffer respectively, and were freeze-dried and purified further on a DEAE-Sephadex A-25 column (0.9 cm × 30 cm) to remove traces of P_i by using a 120 ml gradient of 0.1–0.9 M-tris(hydroxyethyl)ammonium carbonate buffer (pH 7.6). ADP and GDP were eluted from the column at 0.5 M and 0.56 M salt concentrations respectively.

Purification of labelled nucleoside triphosphate

The reaction mixture containing β -labelled nucleoside triphosphate was applied to a DEAE-Sephadex A-25 column (0.9 cm × 30 cm) equilibrated with 100 mM-tris(hydroxyethyl)ammonium carbonate buffer, pH 7.6, and elution was performed by using a 200 ml linear gradient of 0.1–1 M of the same buffer. This column gave a good separation of the triphosphates from the diphosphates. ATP and GTP were eluted from the column at approx. 0.75 M and 0.82 M salt concentrations respectively.

Results and Discussion

The synthesis of β -labelled nucleoside triphosphates has been carried out with both poly(A) and poly(G) as nucleoside donor. The yields at both

reaction steps in each case are shown in Table 1. The reaction with poly(U) and poly(C) has not been performed. Since the latter two polynucleotides can also be phosphorolysed with polynucleotide phosphorylase (Kaufmann *et al.*, 1973), the technique may also be used for the synthesis of β -labelled pyrimidine nucleoside triphosphates. However, since 3-phosphoglycerate kinase is fairly specific for purine nucleotides (Krietsch & Bücher, 1970), another suitable kinase might be used for the conversion of labelled diphosphate into triphosphate.

The specific radioactivity of triphosphates synthesized by this method depends on the specific radioactivity of [³²P]P_i used for phosphorolysis, whereas the extent of phosphorolysis depends on the concentration of P_i. At the concentration used in the present experiments (i.e. 2 mM), more than 60% of the synthetic polynucleotide is converted into the corresponding nucleoside diphosphate (Table 1). However, an almost complete phosphorolysis occurs at a concentration of 20 mM-P_i, but with a 10-fold decrease in the specific radioactivity. The triphosphate produced had a specific radioactivity greater than 5000 mCi/mmol (Table 1). Phosphorolysis takes place with more than 40% yield at 1 mM-P_i concentration (results not shown). This method can therefore be used for the synthesis of triphosphates of even higher specific radioactivity. No decrease in the specific radioactivity occurs in the second reaction if GTP is used to convert ADP into ATP. Complete separation of ATP from GTP and diphosphates was achieved on a DEAE-Sephadex column (Fig. 1).

The formation of 1,3-diphosphoglycerate *in vivo* is coupled to the oxidation of glyceraldehyde 3-phosphate in the presence of NAD⁺ and P_i. In the purified system the highly endergonic reaction resulting in the formation of 1,3-diphosphoglycerate and NADH would not be expected to take place to any appreciable extent if the oxidized coenzyme is not regenerated. In the presence of nucleoside triphosphate, however, an exchange reaction takes place between P_i and the terminal phosphate group of the triphosphate (Glynn & Chappell, 1964) through the formation of 1,3-diphosphoglycerate. 1,3-Diphosphoglycerate thus formed reacts with either

Table 1. *Synthesis of β -labelled nucleoside triphosphate*
The experiments were performed as described in the Experimental section.

Expt. no.	Polynucleotide (μ mol of P)	Nucleoside diphosphate formed (μ mol)	Nucleoside triphosphate formed (μ mol)	Yield (%)	
				Based on polynucleotide	Based on radioactivity
1	Poly(A) (0.5)	0.32	0.21	42	22
2	Poly(A) (0.5)	0.29	0.23	44	23
3	Poly(G) (0.6)	0.34	0.25	41	23
4	Poly(G) (0.6)	0.33	0.23	39	25

Fig. 1. Purification of labelled ATP on DEAE-Sephadex

Chromatography was performed as described in the Experimental section; 2 ml fractions were collected. For radioactivity measurement, 5 μl of each fraction was pipetted on to 1 cm $\times$ 1 cm squares of Whatman DE 81 paper, dried and counted for radioactivity in a liquid-scintillation counter. —, A_{258} (monitored with a LKB Uvicord II spectrophotometer); ----, radioactivity; ····, buffer concn.

Table 2. Cleavage of labelled triphosphate with polynucleotide kinase

Polynucleotide kinase assay was performed as described by Lillehaug & Kleppe (1975). Polynucleotide used as acceptor for the γ -phosphate group was a mixture of oligonucleotides purified from a ribonuclease T_1 digest of RNA isolated from mouse myeloma cells. At the end of the reaction nucleoside diphosphate was removed from oligonucleotides by DEAE-cellulose chromatography as described in the Experimental section. Radioactivity recovered from oligonucleotides is expressed as percentage of radioactivity recovered from nucleoside diphosphate and oligonucleotides.

Expt. no.	Labelled triphosphate	Percentage of radioactivity recovered in the oligonucleotide fraction
1	$[\beta\text{-}^{32}\text{P}]\text{ATP}$	0.005
2	$[\beta\text{-}^{32}\text{P}]\text{ATP}$	0.003
3	$[\beta\text{-}^{32}\text{P}]\text{GTP}$	0.002
4	$[\alpha\text{-}^{32}\text{P}]\text{GTP}$	98.300

ADP or GDP to produce the corresponding triphosphate. In the absence of net synthesis of 1,3-diphosphoglycerate, the extent of conversion of labelled nucleoside diphosphate into triphosphate depends on the concentration of unlabelled triphosphate used to regenerate 1,3-diphosphoglycerate. At the relative concentrations of labelled di-

phosphate and unlabelled triphosphate used in these experiments (1:5), 70% of the labelled diphosphate is converted into the corresponding triphosphate. It should be possible to increase the yield at this step further by increasing the concentration of unlabelled triphosphate.

As pointed out by Glynn & Chappell (1964), even if the enzyme preparations used for the second reaction step contain a trace of aldehyde kinase, no detectable exchange of $[\text{P}^{32}]\text{P}_i$ between β - and γ -phosphate groups takes place. Thus no radioactivity could be detected in the γ -phosphate group of the labelled nucleoside triphosphate (Table 2).

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