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A simple method for the preparation of [β-^{32}P]purine nucleoside triphosphates

Yasuhiro Furuichi and Aaron J. Shatkin

Roche Institute of Molecular Biology, Nutley, NJ 07110, USA

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ABSTRACT

A rapid, simple and inexpensive procedure is described for the preparation of purine ribo- and deoxyribonucleoside triphosphates specifically and highly radiolabeled with [^{32}P]phosphate in the β position. The method involves two successive enzymatic reactions: conversion of donor [γ-^{32}P]ATP in the presence of an excess of acceptor 5'-mononucleotide to the 5'-diphosphates by myokinase or guanosine 5'-monophosphate kinase followed by phosphorylation with pyruvate kinase to yield 5'-triphosphates.

INTRODUCTION

Nucleoside triphosphates (NTPs) labeled with [^{32}P]-radioactivity in the β or γ position are useful for studies of nucleic acid biosynthesis since the nascent polynucleotide chains produced in the presence of γ-, β- or [β,γ-^{32}P]NTP contain 5'-terminal $\overset{*}{\text{pppN}}$, $\overset{*}{\text{pppN}}$ or $\overset{**}{\text{pppN}}$, respectively. In addition, specifically radiolabeled NTPs are indispensable for clarifying the mechanisms of various biochemical reactions which involve phosphate release, transfer or cyclization. Glynn and Chappel (1) described a procedure for preparing [γ-^{32}P]NTP from [^{32}P]phosphate and NTP by an enzymatic exchange reaction. This procedure has made it relatively easy to prepare high specific radioactivity [γ-^{32}P]NTPs which are now commercially available at affordable prices. However, a simple method for the preparation of [β-^{32}P]NTPs has not yet been established, and the cost of these compounds from biochemical supply companies is prohibitively high. Several methods for the preparation of [β-^{32}P]NTPs have been published including the use of intact permeable *E. coli* (2), subcellular components of brine shrimp (3), enzymatic phosphorolysis of ribopolymers by purified polynucleotide phosphorylase (4) and chemical synthesis (5); but in order to obtain high specific activity [β-^{32}P]NTP (3000-5000 Ci/mmole) in good yield for studies

on RNA 5'-termini (6), we have devised a new procedure that is rapid, simple and relatively inexpensive. Synthesis of labeled ATP is based upon an equilibration between [γ-^{32}P]ATP and a molar excess of non-radioactive 5'-AMP catalyzed by purified rabbit muscle myokinase. Ribo-GTP, dGDP and rITP were similarly prepared with hog guanosine 5'-monophosphate kinase (GMP-kinase) and [γ-^{32}P]ATP as the [^{32}P] donor. In both reactions, [^{32}P]-phosphate was transferred to the β-position of acceptor 5'-mononucleotides and the resulting [β-^{32}P]NDPs were then completely converted to [β-^{32}P]NTPs by incubation with phosphoenolpyruvate (PEP) and pyruvate kinase (PEP-kinase). The triphosphate products can be used directly for reactions that are not inhibited by 5'-mononucleotides, PEP and PEP-kinase, for example the vaccinia- and reovirus-associated RNA polymerases. For other studies, the radiolabeled triphosphates can be purified by column chromatography as described.

EXPERIMENTAL PROCEDURES

Materials. Myokinase (rabbit muscle, 720 U/ml), guanosine-5'-monophosphate kinase (GMP-kinase from hog brain, 20 U/ml) and pyrophosphatase (yeast, 200 U/ml) were obtained from Boehringer (Mannheim, West Germany). Phosphoenolpyruvate kinase (PEP-kinase from rabbit muscle, 3000 U/ml) and bacterial alkaline phosphatase (BAPF, 100 U/ml) were purchased from Calbiochem and Worthington Biochemical Corp., respectively. P_1 nuclease was purchased from Yamasa Shoyu Co. Phosphoenolpyruvate (PEP) and nucleoside-5'-monophosphates were obtained from Calbiochem. [γ-^{32}P]ATP (specific activity 16.1 Ci/mmole) was from Amersham/Searle, and thin layer PEI cellulose plates (20 cm x 20 cm) were from J.T. Baker Co. (Philipsburg, New Jersey). [^{3}H]CTP (specific activity 25 Ci/mmole) and [^{3}H]UTP (specific activity 16.4 Ci/mmole) were from Schwarz/Mann and Amersham/Searle, respectively.

Methods. 1. Preparation of [β-^{32}P]adenosine triphosphate. Transfer of [^{32}P]-labeled γ phosphate of ATP to the ATP β-position was accomplished in 2 sequential steps: in the presence of 5'-AMP, myokinase catalyzed a transfer reaction between $\overset{*}{\text{p}}$ppA and pA to form $\overset{*}{\text{p}}$pA which was then converted to $\overset{*}{\text{p}}$ppA by incubation with PEP and PEP-kinase. In the first reaction, the incubation mixture (50 µl) contained 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM cysteine, 10 nmoles ATP including 0.5 µCi [γ-^{32}P]ATP, increasing amounts (50-2000 nmoles) of 5'-AMP (or 5'-dAMP), and 0.72 U of myokinase. After 30 min at 30°C, 1 µl of the mixture was spotted on PEI cellulose and chromatography was carried out in 1 M LiCl (7). For the large scale preparation of [β-^{32}P]-ATP, 1 mCi of undiluted [γ-^{32}P]ATP (62 nmoles) was used in a reaction mix-

ture (0.3 ml) containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM cysteine (freshly prepared), 6.2 µmoles 5'-AMP and 4.2 U myokinase. Following incubation (30°C - 30 min), the reaction mixture was extracted twice with an equal volume of phenol and the residual phenol was removed from the aqueous phase by ether extraction. For the second step, conversion of the [β-^{32}P] ADP product (∿60 nmoles) to [β-^{32}P]ATP, the solution was adjusted to 0.5 ml containing final concentrations of 80 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM PEP, 7.5 U PEP-kinase, and residual unreacted 5'-AMP (∿60 µmoles). After incubation at 37°C for 30 min, an aliquot of the mixture was removed for analysis by PEI thin layer chromatography and the remainder was either lyophilized or frozen for later use.

2. Preparation of [β-^{32}P]guanosine triphosphate. Using sequential reactions with GMP kinase and PEP-kinase, 0.9 mCi of [β-^{32}P]GTP was obtained from 1 mCi [γ-^{32}P]ATP and 5'-GMP. The first incubation mixture (0.2 ml) contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mCi [γ-^{32}P]ATP (62 nmoles), 1 µmole 5'-GMP and 0.04 U GMP-kinase. After 30 min at 37°C, the mixture was extracted with equal volumes of phenol (2 times) and H_2O-saturated ether. Residual ether was evaporated with a stream of air, and the conversion of [β-^{32}P]GDP to GTP was then carried out as for ADP. In brief, the reaction mixture was adjusted to pH 7.6-8 and a volume of 0.3 ml by addition of the following final concentrations of reagents for phosphorylation: 60 mM Tris-HCl (pH 7.6-8), 15 mM $MgCl_2$, 16 mM PEP and 9 U PEP-kinase. After 30 min at 37°C, the sample was frozen at -70°C.

3. Messenger RNA synthesis in vitro by vaccinia- and reovirus-associated RNA polymerases with [β-^{32}P]-labeled nucleoside triphosphates. Purified vaccinia virus (8) and reovirus (9) were prepared as described. It has already been shown that reovirus mRNA synthesized in vitro in the presence of the methyl donor, S-adenosylmethionine (Adomet) contains 5'-terminal m^7GpppG^m-C... (6) and that addition of both pyrophosphate and the methylation inhibitor, S-adenosylhomocysteine (Adohcy) to reaction mixtures yields mRNAs with unblocked 5'-ppG-C... (10). Reovirus mRNA containing 5'-ppG-C... was synthesized with [β-^{32}P]GTP in a reaction mixture (0.4 ml) containing 50 mM Tris-HCl (pH 8), 7 mM $MgCl_2$, 2 mM ATP, 2 mM CTP, 0.2 mM GTP, 0.5 mM UTP including 40 µCi [^{3}H]UTP, 0.5 mM sodium pyrophosphate, 0.25 mM Adohcy, 5 mM PEP, 6 U PEP-kinase, 500 µg reovirus cores and 33 µCi [β-^{32}P]GTP. The [β-^{32}P]GTP was used directly from the PEP-kinase incubation step, after concentrating the reaction mixture from 0.3 to 0.2 ml by evaporation with a

stream of nitrogen. Incubation for RNA synthesis was for 2.5 hr at 45°C. Reovirus cores were then removed by centrifugation (1000 xg, 15 min, 4°C) and viral mRNA labeled with [^{32}P] and [^{3}H] was isolated from the supernatant by phenol extraction and gel filtration in Sephadex G-100.

Vaccinia mRNA was synthesized in the presence of [β-^{32}P]ATP, Adomet, and 0.1 U/ml of inorganic pyrophosphatase [E.C. 3.6.1.1], conditions that favor the formation of viral mRNA with a 5'-cap structure, m^{7}GpppN... (11). The incubation mixture (0.4 ml) contained 60 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 0.8 mM ATP, 1.5 mM CTP including 40 μCi [^{3}H]CTP, 0.8 mM GTP, 2 mM UTP, 0.23 mM Adomet, 25 mM dithiothreitol (DTT), 7 mM PEP, 9 U PEP-kinase, 0.04 U yeast inorganic pyrophosphatase, vaccinia cores prepared from 600 μg purified virus by non-ionic detergent treatment and 100 μCi [β-^{32}P]ATP prepared as described. After 1 hr at 37°C, virus was removed by centrifugation (1000 xg, 15 min, 4°C) and mRNA was isolated by phenol extraction and gel filtration.

4. Chromatography and paper electrophoresis. Column chromatography on Dowex-1 anion exchange resin and chromatography and electrophoresis on Whatman 3MM paper were performed as described previously (12,13).

5. Purification. To obtain [β-^{32}P]NTP free from 5'-mononucleotides and other reagents present in the incubation mixture, samples were diluted 5-fold with H_2O after the second reaction and applied onto a 1x7 cm column of DEAE cellulose (DE-52) with or without prior extraction with phenol to inactivate PEP-kinase. The column was washed sequentially with 20 ml each of 50 mM and 0.1 M ammonium bicarbonate. 5'-Mononucleotides eluted in 0.1 M ammonium bicarbonate, and the radioactive NTP was then recovered by elution in 1 M ammonium bicarbonate which was volatilized by lyophilization. Further purification can be achieved by chromatography on Dowex-1 resin as described in Fig. 2.

RESULTS AND DISCUSSION

Preparation of [β-^{32}P] Purine Ribonucleoside Triphosphates.

(i) [β-^{32}P]ATP. Muscle myokinase [E.C. 2.7.4.3] catalyzes the transfer of the γ-phosphate of ATP to 5'-AMP, producing two molecules of ADP by the reaction: $\overset{*}{p}ppA + pA \xrightleftharpoons{\text{Myokinase}} ppA + \overset{*}{p}pA$.

In the presence of an excess of AMP, the equilibrium shifts in favor of the formation of ADP. If the phosphate donor is [γ-^{32}P]ATP, one of the resulting molecules of ADP is [^{32}P]-labeled in the β position. Fig. 1A shows the results of an experiment in which the effect of AMP concentration on the formation of ADP was studied. Increasing levels of AMP (50 to 2000 nmoles) were added to a reaction mixture containing 10 nmoles of [γ-^{32}P]ATP and myokinase. After incubation, the products were analyzed by PEI thin layer

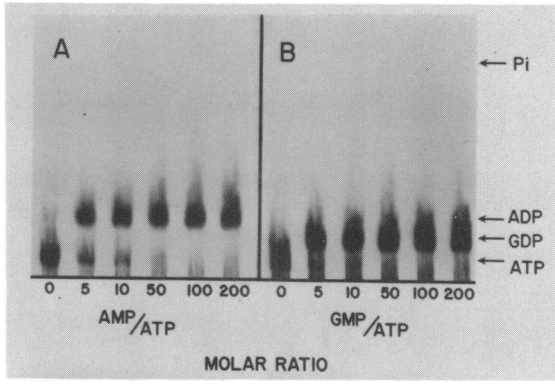

Figure 1: Effect of 5'-mononucleotide/[γ-^{32}P]ATP ratio on the formation of [β-^{32}P]-labeled 5'-nucleoside diphosphates. Reaction mixtures containing 10 nmoles [γ-^{32}P]ATP (0.5 μCi) were incubated for 30 min as desscribed in the methods section with myokinase and 5'-AMP at 30°C (A) or with GMP-kinase and 5'-GMP at 37°C (B). Reactions were stopped by chilling on ice and 1 μl was removed for analysis on PEI cellulose thin layer plates. ADP, GDP and ATP added as standard markers were located under ultraviolet light and autoradiograms of the dried plates were obtained with Kodak X-ray film #BB-5.

chromatography (7). As the AMP/ATP molar ratio was increased, the transfer of radioactive phosphate from the γ to β position increased concomitantly. For example, in the presence of a 5-fold excess of AMP, the conversion of [^{32}P] phosphate from [γ-^{32}P]ATP to [β-^{32}P]ADP was 67% complete, and with a 100-fold excess of AMP 98% of the total [^{32}P] radioactivity was transferred. There was little or no release of [^{32}P] inorganic phosphate (Pi) from the radioactive nucleotides, indicating that the myokinase contained no phosphatase [E.C. 3.1.3.1] or nucleotide pyrophosphatase [E.C. 3.1.4.1] capable of degrading [γ-^{32}P]ATP or [β-^{32}P]ADP.

The products of a myokinase-containing reaction mixture with an AMP/ATP molar ratio of 200 were also analyzed by Dowex-1 column chromatography. Most of the [^{32}P]radioactivity eluted in the position of ADP with only a small amount (<1% of total [^{32}P]) in the position of the starting material, ATP (Fig. 2A). After pooling and desalting the appropriate fractions, the ADP was isolated free of ATP and 5'-AMP as described in the Experimental section. The purified [β-^{32}P]ADP was converted to [β-^{32}P]ATP by incubation with PEP and PEP kinase [E.C. 2.7.1.40]. Conversion of ADP was quantitative as shown by column chromatography of the products on Dowex-1 (Fig. 2B).

Although the two separate reactions which yielded [β-^{32}P]ATP from [γ-^{32}P]ATP proceeded quantitatively under the selected conditions, it was

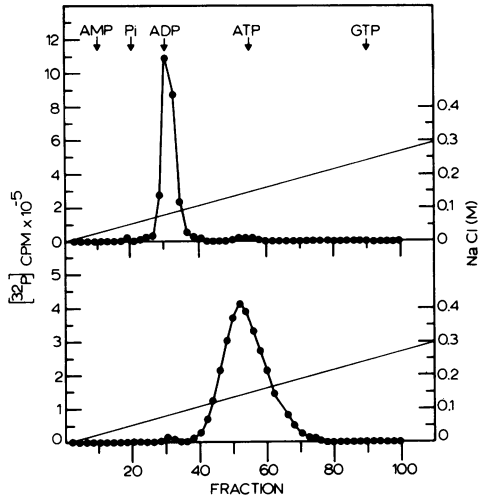

Figure 2: Characterization of [β-^{32}P]ADP and ATP by Dowex-1 anion exchange column chromatography. A. After the myokinase reaction with an AMP/ATP molar ratio of 200, the incubation mixture was phenol-extracted, diluted 5-fold with water and applied to a column (0.6 x 25 cm) of Dowex-1 X8 resin equilibrated with 0.01 M HCl. A linear gradient of 0 to 0.4 M NaCl in 0.01 HCl (total = 400 ml) was applied and 2 ml fractions were collected and assayed by Cerenkov radiation in a scintillation counter. Peak fractions (25-28) were pooled, desalted and lyophilized. The purified [β-^{32}P]ADP was then converted to [β-^{32}P]ATP by incubation with PEP and PEP-kinase as described in methods. B. The products of the PEP-kinase reaction were similarly analyzed by Dowex-1 chromatography.

time-consuming to isolate the intermediate [β-^{32}P]ADP by column chromatography. In addition, recovery of the radioactive compound was incomplete due to losses during the desalting step. To avoid these difficulties, we proceeded directly from the myokinase to the PEP-kinase reaction without isolating the [β-^{32}P]ADP intermediate. The myokinase was inactivated after the first reaction by extraction with phenol, and following removal of phenol with ether, the second reaction, $\overset{*}{\text{ppA}} \xrightarrow{\text{PEP \& PEP kinase}} \overset{*}{\text{pppA}}$, was done. Fig. 3 shows the successive conversion of [γ-^{32}P]ATP (lane c) to [β-^{32}P]ATP (lane e) via [β-^{32}P]ADP (lane d). Apparently, the presence in the first incubation mixture of 20 mM 5'-AMP (a 200-fold excess for quantitative transfer of the γ phosphate of ATP to AMP) did not inhibit the action of the PEP-kinase in converting [β-^{32}P]ADP to [β-^{32}P]ATP in the second step.

Incubation mixtures for the in vitro synthesis of DNA or RNA often include PEP and PEP-kinase in order to recycle the NDPs derived from dephosphorylation of NTPs back to the triphosphate level. For instance, optimal

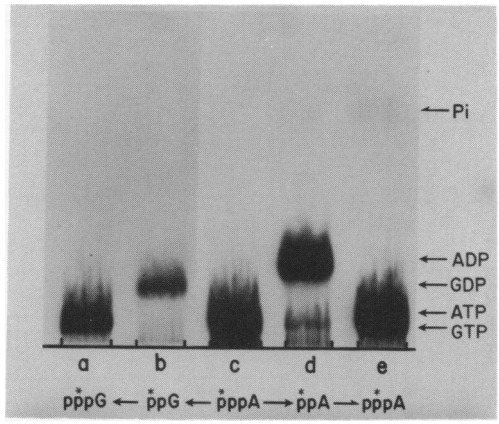

Figure 3. Preparation of [β-^{32}P]ATP and [β-^{32}P]GTP from [γ-^{32}P]ATP without isolating nucleoside diphosphate intermediates. Autoradiographs of thin layer chromatograms show the products of two successive reactions starting with [γ-^{32}P]ATP (c) which was converted to [β-^{32}P]ADP by myokinase in the presence of an AMP/ATP molar ratio of 100 (d) and then to [β-^{32}P]ATP by PEP and PEP-kinase (e). For [β-^{32}P]GDP formation from [γ-^{32}P]ATP by GMP-kinase, the GMP/ATP molar ratio was 100 (b) and the subsequent conversion to [β-^{32}P]-GTP was quantitative (a).

synthesis of viral mRNAs by reovirus cores or vaccinia virus requires the addition of a NTP regenerating system because these particles contain, in addition to RNA polymerase, a potent nucleotide phosphohydrolase activity which degrades NTPs to NDPs (14-16). Because the presence of PEP and PEP-kinase does not interfere with the incorporation of [β-^{32}P]ATP into nucleic acids and 5'-AMP at a level of 2.5 mM did not markedly inhibit reovirus- and vaccinia virus-associated RNA polymerase activities (<10%, unpublished results), for reactions catalyzed by these enzymes we could eliminate the procedures for isolation of the [β-^{32}P]-labeled intermediates and products and thus obtain higher recoveries. (Other polymerase systems may require purification of the [^{32}P] products before use.) Conversion of [γ-^{32}P]ATP to [β-^{32}P]ATP by the successive 2-step procedure can be completed in 90 min with 90% of the [^{32}P] counts recovered as [β-^{32}P]ATP. To avoid the small losses (<10%) suffered during phenol extraction, we tried to inactivate the myokinase without phenol treatment by heating the reaction mixture (100°C, 1 min), adding N-ethyl maleimide (NEM) in a 2-fold excess over the reducing agent in the reaction mixture, or using both treatments. However, these procedures did not inactivate myokinase and phenol extraction was necessary. [β-^{32}P]ATP lyophilized directly from the second reaction mixture was utilized as substrate for vaccinia virus mRNA synthesis *in vitro* (11). In order to test whether

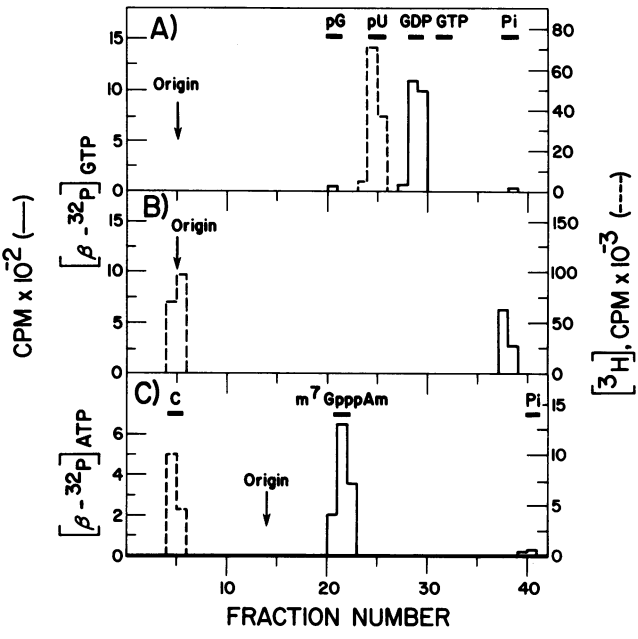

Figure 4. Characterization of 5'-terminal structures of mRNAs synthesized by reovirus- and vaccinia-virus associated RNA polymerases with unpurified [β-^{32}P]GTP and -ATP as precursors. Conditions for the synthesis and isolation of [β-^{32}P]-labeled mRNAs were as described in methods. Samples were digested and analyzed by electrophoresis on Whatman 3MM paper at 53 V/cm for 60 min in 10% pyridine-acetate buffer, pH 3.5. A. Reovirus mRNA containing presumptive 5'-terminal $\overset{*}{p}$pG and internally located [^{3}H]U (54 µg, 3000 [^{32}P] cpm, 8.7 x 10^{6} [^{3}H] cpm) was digested with 10 µg P_1 nuclease in 50 µl of 10 mM sodium acetate buffer pH 6.0 for 20 min at 37°C (6). B. Reovirus mRNA labeled as in A (22 µg, 1200 [^{32}P] cpm, 3.5 x 10^{6} [^{3}H] cpm) was digested with 1 U/ml BAPF for 20 min at 37°. C. Vaccinia virus mRNA with presumptive 5'-structures $m^{7}Gp\overset{*}{p}pA^{m}$ and internal [^{3}H]C (∿30 µg, 1500 [^{32}P] cpm, 3.0 x 10^{5} [^{3}H] cpm) was digested successively by P_1 nuclease and BAPF as in A and B.

[β-^{32}P]ATP was incorporated at the 5'-termini of the RNA, vaccinia virus mRNA synthesized in the presence of [β-^{32}P]ATP and Adomet was isolated from the reaction mixture, digested with P_1 nuclease and alkaline phosphatase and analyzed by paper electrophoresis. Most of the [^{32}P] (97%) incorporated into the RNA was found in the 5'-terminal "cap" structure, $m^{7}G\overset{*}{p}ppA^{m}$ (Fig. 4C). The rest of the [^{32}P] migrated in the position of inorganic phosphate and presumably was derived from 5'-$\overset{*}{p}$pA-termini resulting from incomplete capping of the nascent chains. These data confirm that the [β-^{32}P]ATP obtained from the phosphorylation reaction is incorporated into 5'-ends of RNA and in this system can be used without purification.

(ii) [β-^{32}P]GTP. The same principles and a different enzyme were applied for the preparation of [β-^{32}P]GTP by the reaction:

$$\overset{*}{\text{pppA}} + \text{pG} \xrightarrow{\text{GMP-kinase}} \text{ppA} + \overset{*}{\text{pp}}\text{G} \xrightarrow{\text{PEP \& PEP-kinase}} \text{pppA} + \overset{*}{\text{ppp}}\text{G}.$$

GMP-kinase [E.C. 2.7.4.8] catalyzes the transfer of γ-phosphate of ATP to 5'-GMP, yielding 5'-GDP. This reaction, unlike that catalyzed by myokinase, is irreversible; consequently the products are exclusively ADP and GDP. Since the transfer of phosphate occurs between heterologous nucleotide species, the specific radioactivity of the product [β-^{32}P]GDP is identical to the donor [γ-^{32}P]ATP, in contrast to the myokinase reaction where the specific radioactivity in the $\overset{*}{\text{pp}}$A is decreased 2-fold by formation of an equal amount of ppA from $\overset{*}{\text{ppp}}$A. (It should be noted that [γ-^{32}P]ATP of specific activity 5000 Ci/mmole is commercially available.) When [γ-^{32}P]ATP was incubated with increasing amounts of 5'-GMP in the presence of GMP-kinase, [^{32}P]GDP was formed (Fig. 1B). The efficiency of [^{32}P]phosphate transfer increased at higher GMP/ATP ratios. At a molar ratio of 50, more than 90% of the [^{32}P] counts in ATP were transferred to a compound which migrated with standard marker GDP in PEI cellulose chromatography. At a ratio of 100, the [^{32}P] transfer was essentially complete. In order to characterize the products further, aliquots of a reaction mixture (GMP/ATP=50) were analyzed by paper chromatography after the first (GMP-kinase) and second incubations (phosphorylation with PEP and PEP-kinase). The sample obtained from the first step yielded a strong spot in the position of the standard marker GDP, consistent with $\overset{*}{\text{pp}}$G as the major product (Fig. 5, lane b). The small amount (8%) of [^{32}P]-containing material migrating between GTP and GDP probably is unreacted [γ-^{32}P]ATP. The chromatogram of the products from the second incubation mixture (Fig. 5, lane a) shows that the $\overset{*}{\text{pp}}$G was quantitatively converted to $\overset{*}{\text{ppp}}$G. Again, there was no significant amount of [^{32}P] inorganic phosphate released during the two successive reactions.

The sequence of reactions for the preparation of [β-^{32}P]GTP from [γ-^{32}P]-ATP can be derived directly by examination of autoradiographs of the reaction products (Fig. 3). In the first reaction the γ-phosphate of ATP was transferred to the β-position of GDP (lane b) which was directly converted to GTP by addition of the components of the second incubation (lane a).

[β-^{32}P]GTP was utilized for *in vitro* mRNA synthesis by reovirus cores incubated under conditions that yielded mRNA products with 5'-terminal ppG-C... as described in the experimental section. [β-^{32}P]GTP added directly from the phosphorylation reaction mixture to the components for mRNA synthesis was efficiently incorporated into mRNA at a reasonable level for 5'-end labeling

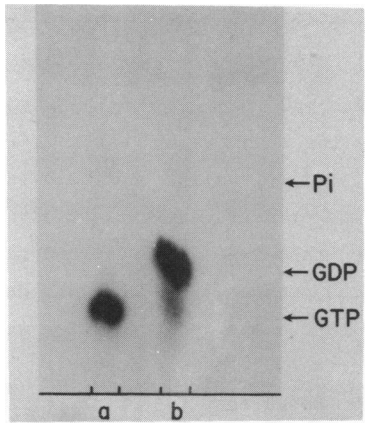

Figure 5. Characterization of [β-^{32}P]GDP and [β-^{32}P]GTP by paper chromatography. Reaction products obtained from incubation mixtures containing GMP-kinase and a GMP/ATP ratio of 50 were analyzed by decending paper chromatography on Whatman 3MM paper with isobutyric acid - 0.5 M NH_4OH (10:6 v/v) as solvent. The [β-^{32}P]GDP shown in lane b was converted to [β-^{32}P]GTP by PEP-kinase (lane a).

(1 mole [^{32}P]/2800 nucleotides). When the RNA products were treated with E. coli alkaline phosphatase and the digests analyzed by electrophoresis, all the [^{32}P] counts migrated in the position of Pi (Fig. 4B). The [^{3}H] counts incorporated from [^{3}H]UTP into internal positions, on the other hand, remained at the origin as RNA. After hydrolysis with P_1 nuclease, most of [^{32}P] counts migrated to the position of 5'-GDP while all the [^{3}H] counts were released as 5'-UMP (Fig. 4A). These results together with those obtained with vaccinia virus mRNA clearly indicate that [β-^{32}P]ATP and [β-^{32}P]GTP can be satisfactorily used directly for in vitro RNA synthesis by virion polymerases, although further purification by column chromatography may be necessary for other systems.

Characterization of [β-^{32}P]ATP and [β-^{32}P]GTP.

In order to obtain additional evidence that the [^{32}P]-containing ATP and GTP synthesized as described above were labeled exclusively in the β position the following experiments were carried out. Phenol-extracted reaction mixtures containing presumptive $\overset{*}{p}pA$, $\overset{*}{p}ppA$, $\overset{*}{p}pG$ and $\overset{*}{p}ppG$ were incubated with reovirus cores, which contain γ-phosphatase (nucleotide phosphohydrolase; 14,15) or with E. coli alkaline phosphatase. The digests were analyzed by PEI cellulose chromatography. The results in Fig. 6 demonstrate that [^{32}P]-labeled phosphates are located only in the β-position of the nucleotide products. In panel A, the untreated presumptive [β-^{32}P]ADP migrated with marker ADP (lane

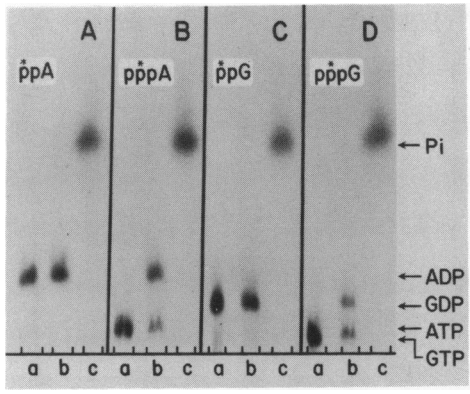

Figure 6. Verification that the β-position is [^{32}P]-labeled in the nucleoside di- and triphosphate products. Presumptive [β-^{32}P]-labeled ADP, ATP, GDP and GTP were incubated at a concentration of 0.6 nmoles/25 µl as follows: (a) in water only; (b) with purified reovirus cores containing γ-phosphatase; and (c) with bacterial alkaline phosphatase (BAPF). Incubation mixtures with reovirus cores contained 25 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, and 200 µg washed cores prepared as described previously (13). After 30 min incubation at 37°C, reaction mixtures were chilled, centrifuged (1000 x g - 15 min) to remove cores, and 2 µl samples were analyzed by PEI cellulose chromatography. Reactions with bacterial alkaline phosphatase were done similarly except that viral cores were replaced by 0.02 unit BAPF. All [β-^{32}P]-labeled nucleotides were obtained directly from the phenol-extracted first or second reaction mixture and used without further purification. [β-^{32}P]ADP and ATP were from mixtures containing a molar ratio of AMP/ATP = 100; for [β-^{32}P]GDP and GTP, the GMP/ATP ratio was 50. Standard markers ADP, GDP, ATP and GTP were included in the chromatography.

a), remained unchanged after incubation with reovirus cores (lane b), and yielded [$^{32}P_i$] following treatment with alkaline phosphatase (lane c). By contrast, panel B shows that after treatment of [^{32}P]-labeled presumptive ṗpṗpA with reovirus cores, much of the radioactivity shifted to the position of ADP with no release of [^{32}P] inorganic phosphate. Again, all of the [^{32}P]-labeled nucleotide phosphates were susceptible to phosphatase digestion. These results unequivocally demonstrate that the site of [^{32}P]-labeling in ATP is the β-position. The presumptive [β-^{32}P]GDP and [β-^{32}P]GTP were similarly characterized as shown in panels C and D of Fig. 6. In particular, the results with GTP clearly indicate that the β-position is [^{32}P]-labeled since the phosphate group removed by the reovirus core-associated γ-phosphatase is non-radioactive (panel D, lane b).

Preparation of Other [β-^{32}P]-labeled Purine Nucleotides.

(i) [β-^{32}P]dADP and [β-^{32}P]dGDP. Although DNA polymerases apparently require primers for the de novo initiation of DNA synthesis (17,18) and con-

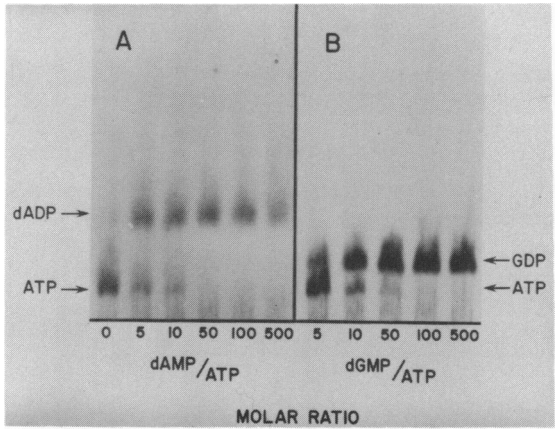

Figure 7. Formation of [β-^{32}P]-labeled dADP and dGDP. A. Reaction mixtures (25 µl) containing 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 nmole [γ-^{32}P]ATP (0.25 µCi), increasing amounts of deoxyadenosine-5'-monophosphate (5-500 nmoles) and 2.1 U myokinase were incubated at 30°C for 30 min. B. A mixture containing 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 nmole [γ-^{32}P]ATP (0.5 µCi), deoxyguanosine-5'-monophosphate (5-500 nmoles) and 0.01 U GMP-kinase was incubated at 37°C for 30 min. The products from both incubations were analyzed by PEI-cellulose chromatography.

sequently nascent chains presumably would not be labeled by using [β-^{32}P]dNTP precursors, it is also important to establish a simple method for preparing [β-^{32}P]-labeled deoxyribonucleoside triphosphates for other types of DNA studies. [β-^{32}P]-labeled dADP and dGDP were made as above by using myokinase and GMP-kinase, respectively. Both deoxyribonucleoside diphosphates could be obtained from [γ-^{32}P]ATP and the corresponding 5'-deoxyribomononucleotide (Fig. 7). The transfer of phosphate from [γ-^{32}P]ATP to dAMP or dGMP appears to be slightly less efficient than observed for the corresponding ribonucleoside monophosphate, but at dAMP/ATP or dGMP/ATP ratios of 100, transfer was almost quantitative. We have not carried out the phosphorylation reaction with the [β-^{32}P]-labeled dADP and dGDP, but PEP-kinase is known to phosphorylate deoxy- as well as the ribose-containing compounds.

(ii) Preparation of [β-^{32}P]ITP. The GTP analogue, ITP, has been used for *in vitro* RNA synthesis (19), and [β-^{32}P]ITP can be prepared by the direct deamination of [β-^{32}P]ATP by nitric acid. However, this procedure requires tedious purification to remove unreacted ATP and chemicals which might inhibit subsequent biochemical reactions. Therefore, we have applied the same enzymatic procedures for the preparation of [β-^{32}P]ITP. [β-^{32}P]IDP

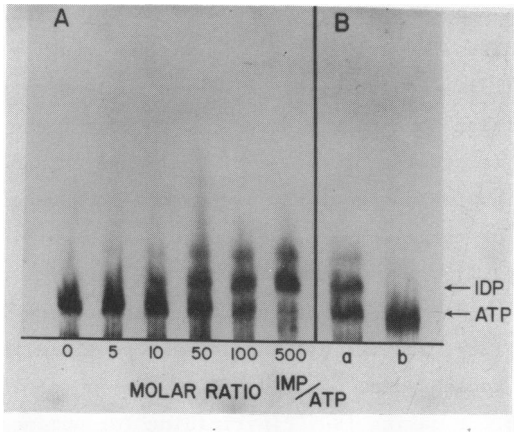

Figure 8. Preparation of [β-^{32}P]IDP and ITP from [γ-^{32}P]ATP. A. Reaction mixtures (50 µl) containing 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 nmole (0.5 µCi) [γ-^{32}P]ATP, inosine-5'-monophosphate (5-500 nmoles) and 0.02 U GMP-kinase were incubated at 37°C for 30 min, and the reaction products were analyzed by PEI cellulose chromatography. B. A reaction mixture containing a ratio of IMP/ATP=100 was sequentially extracted with phenol and ether as described in methods. The aqueous solution which contained [β-^{32}P]IDP was further incubated with 50 mM Tris-HCl (pH 7.6-8), 12 mM $MgCl_2$, 10 mM PEP and 3 U PEP-kinase at 37°C for 30 min. An aliquot (2 µl) of the mixture was analyzed by PEI cellulose chromatography. In tracks (a) and (b) are shown the products of the first and second reactions, respectively.

is simply and quickly made as described for other purines since GMP-kinase uses IMP as substrate (Fig. 8). However, the efficiency of transfer of γ-phosphate to IMP is much lower than with other purine nucleotides, and thus a higher level of IMP is required to obtain reasonable yields of β-labeled diphosphate product (panel A). [β-^{32}P]IDP, formed by GMP-kinase in an incubation mixture containing a molar ratio of IMP/ATP=100 (panel B, lane a), was quantitatively converted to the triphosphate by PEP-kinase (panel B, lane b). Since the quantitative conversion of [γ-^{32}P]ATP to [β-^{32}P]IDP requires a large excess of 5'-IMP (IMP/ATP≈500), it is not possible to utilize [β-^{32}P]-ITP for RNA synthesis without purification.

(iii) [β-^{32}P]pyrimidine nucleoside triphosphates. Recent studies on the structure of eukaryotic mRNAs revealed that mammalian cell mRNAs contain pyrimidine as well as purine nucleotides in the 5'-penultimate position, N in the "cap" structure m^7GpppN- (20-25). Cell-free studies of RNA polymerase from both prokaryotic and eukaryotic cells, on the other hand, have led to the generally accepted (but possibly incorrect) notion that RNA synthesis is initiated by purine but not pyrimidine nucleotides (26,27). How, then, is a

pyrimidine-containing cap generated? Processing of primary transcripts may be involved or mechanisms may exist in vivo for generating pyrimidine-initiated nascent RNAs (13). Radioactive pyrimidine nucleoside triphosphates labeled in the β-position have rarely been used to test these possibilities. We were unsuccessful in attempts to prepare pyrimidine triphosphates with the kinases that yielded purine nucleotides, presumably because of the strict substrate specificities of these enzymes. Although not commercially available, dCMP-CMP-UMP-kinase (2.7.3.14), which catalyzes the transfer of γ-phosphate from ATP to dCMP, CMP and UMP has been reported and purified from prokaryotic and eukaryotic cells (28), and its use should make possible the preparation of [β-^{32}P]pyrimidine nucleotides.

Another approach for making [β-^{32}P]pyrimidine (or purine) NTPs is the utilization of the phosphorolysis reaction or the exchange reaction between [$^{32}P_i$] and NDP (29) mediated by polynucleotide phosphorylase. The commercially available enzyme phosphorylyzes ribopolymers to nucleoside diphosphates by the reaction: $pNp(Np)\ N + Pi \rightarrow pNp(Np)_{n-1}N + ppN$. In the presence of radioactive inorganic phosphate-[^{32}P], [β-^{32}P]ppN is released, but the yield is usually less than 20% of the input Pi (L. Phillipson, personal communication). The accumulated ppN can be converted to the triphosphate by PEP-kinase. By this method, or the exchange reaction between NDP and [$^{32}P_i$] (30), [β-^{32}P]-labeled ribonucleotides can be obtained from the appropriate substrates but in lower yield and reduced specific radioactivity as compared to the procedures described in this report.

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