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A "*Cleanup Procedure*" involving periodate oxidation in the enzymatic synthesis of chemically pure α-^{32}P and α-^{33}P labelled deoxyribonucleotides

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Abstract

Enzymatic synthesis of α-^{32}P and α-^{33}P labelled deoxyribonucleotides involves the transfer of radiolabelled phosphorus from either γ-^{32}P adenosine triphosphate (γ-ATP) or γ-^{32}P guanosine triphosphate (γ-GTP). Subsequent removal of these ribonucleotides is essential for the preparation of chemically pure deoxyribonucleotides. Agarose-phenyl boronate columns, which bind specifically to *cis*-diol moieties, have been used for the removal of ribonucleotide contaminants. However, this involves column losses and additional radiation exposure. In the present work we describe a chemical method for the improvement of the chemical purity, based on the preferential oxidation of ribose sugars by periodate. The *cis*-diol moiety of ribose is specifically oxidised to the dialdehyde. The excess periodate ions were destroyed using ethylene glycol. The phosphate group was then cleaved by β-elimination using alkali. The product was purified using anion exchange chromatography. The efficiency of the process was validated using tracer γ-^{32}P ATP and α-^{32}P dATP. Samples at various steps were analysed by TLC, autoradiography and HPLC. During the process ATP is oxidised whereas 2′-deoxyadenosine triphosphate (dATP) remains intact. The α-^{32}P dATP synthesized by this process was assayed for its incorporation in λ-DNA by the random priming method and was found to be effectively incorporated. The process developed is an efficient and convenient method for the preparation of chemically pure deoxyribonucleotides.

Keywords: Ribonucleotides; Deoxyribonucleotides; Periodate oxidation; β-Elimination

1. Introduction

Enzymatic synthesis of ^{32}P and ^{33}P labelled nucleotides is routinely carried out in our laboratory. The synthesis of α-^{32}P/^{33}P dATP involves the use of γ-^{32}P/^{33}P ATP or γ-^{32}P/^{33}P GTP as the radioactive phosphorus donor (Walseth and Johnson, 1979). Since, ATP, GTP and dATP have identical retention times in anion exchange chromatography, it is essential to remove ATP or GTP from the product before purification. γ-^{32}P/^{33}P ATP or γ-^{32}P/^{33}P GTP are the precursors for labelled dATP during the enzymatic synthesis. The synthesis of γ-^{32}P/^{33}P ATP/GTP involves the substrate level phosphorylation of 5′-ADP with labelled ortho-phosphoric acid. The reaction mixture also contains

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β-nicotinamide adenine dinucleotide (β-NAD). 5′-ADP and β-NAD are the primary sources for ribose contamination. Excess ADP present is subsequently converted to ATP. β-NAD being unstable degrades to AMP during the course of the reaction and this is also converted to ATP. Thus, there is a need for destroying the ATP produced during the course of the reaction to obtain chemically pure deoxyribonucleotides. Procedures currently in use involve separating the ribose moieties by use of agarose-phenyl boronate columns based on their affinity for ribose moieties. Deoxyribonucleotides do not bind to the column and hence can be separated (Weith et al., 1970; Hageman and Kuehn, 1977). However, boronate columns are not totally specific to ribonucleotides and have a small interaction with the deoxyribonucleotides. This results in reduction in the overall yield of the product.

Periodate is a widely used reagent for the oxidation of *cis*-diols. Various reviews have been published on the mechanism of periodate oxidation (Fatiadi, 1974, 1981; Sklarz, 1967). Periodate oxidation has been employed in a wide variety of biochemical applications such as RNA sequencing (Whitfeld and Markham, 1953; Neu and Heppel, 1964; Keith and Gilham, 1974). Palmer and Cox (1994) have reported a method for the determination of cellular deoxynucleotide triphosphate content, based on periodate oxidation. This was to remove the interfering ribose nucleotides. Similar work has been reported by other groups (Garrett and Santi, 1979; Hakam et al., 1984; Tanaka et al., 1984; Piall et al., 1986; Andersson, 1988; Decosterd et al., 1999). Periodate has also been used in the derivatisation of polysaccharides and glucose by converting the 1,2-dihydroxyl groups to dialdehyde (Kim et al., 2000). The periodate oxidation of nucleosides and nucleotides and their application in affinity modification of proteins was reviewed by Ermolinskii and Mikhailov (2000). Periodate cleavage of adenosyl group and its elimination at alkaline pH was used in the synthesis of adenosine 5′-^{35}S-(2thiotriphosphate) (Ho and Frey, 1984). Periodate oxidation has also been employed in the synthesis of ^{14}C labelled radioactive phosphatidyl choline (Itabe et al., 2000). More recently, an LC MS assay for intracellular deoxyribonucleotides in presence of inhibiting ribonucleotides was developed involving the removal of the ribose impurities by periodate oxidation (Hennere et al., 2003). Periodate oxidation has also been used in a simple RNA-ligation procedure (Kurata et al., 2003) Therefore, it would be appropriate to use periodate oxidation to remove the ribose moieties, without loss of deoxynucleotide product, when synthesis of radiolabelled deoxynucleotides is carried out at μM scales. In the present work we report a chemical "cleanup" method for improving the chemical purity of the radiolabelled deoxyribonucleotides, using a method based on periodate oxidation.

2. Experimental

Sodium metaperiodate, potassium phosphate and ethylene glycol were obtained from E-Merck, Germany. ATP and dATP were obtained from Sigma Chemicals, St. Louis, Mo, USA. DEAE-Sephadex A-25 resin was obtained from Pharmacia Biotech. Pvt. Ltd., USA. HPLC grade methanol and acetonitrile were obtained from Qualigens, India. Triethylamine was obtained from Qualigens, India. γ-^{32}P ATP and α-^{32}P dATP were prepared in the laboratory. High purity water obtained from MilliQ Academic system (Millipore, USA) was used for all the experiments.

ATP (10 mM), dATP (10 mM) and $NaIO_4$ (10 mM) stock solutions were prepared in water on the day of the experiment. Triethylammonium bicarbonate (TEAB) was prepared in the laboratory as 1 M stock (pH 8.0).

HPLC was carried out using a JASCO HPLC system composed of a JASCO Pu 1580i gradient pump, equipped with a diode array detection (JASCO, MD-1515) system. System control, data acquisition and data analysis were performed using JASCO-BORWIN chromatography software, version 1.5. For sample loading a Rheodyne valve (model-9725i) was used fitted with 20 μl sample loop. The separations were carried out on a HiQ Sil C-18 (250 × 4.6 mm, 5 μm) reverse- phase column (Kyatech Corporation, Japan).

All the experiments were carried out in triplicate to ensure reproducibility.

2.1. Radiotracer analysis

The experimental procedure involved three main steps:

(1) Oxidation of ribose moieties using periodate, at room temperature for 20 min.
(2) Removal of excess periodate, using ethylene glycol.
(3) β-elimination in alkaline condition to cleave the phosphate group and to generate the free base.

ATP (100 nmol, containing 3.7 MBq of γ-^{32}P ATP), dATP (100 nmol, containing 3.7 MBq of α-^{32}P dATP) and a mixture of these two were used as the three test samples. The total reaction volume in each case was 1 ml. The reaction was initiated by adding 1 μmol of periodate. The periodate oxidation step was carried out at room temperature for 20 min. Excess periodate was destroyed by the addition of 100 μmol of ethylene glycol. The subsequent alkali hydrolysis step was carried out at 37 °C for 30 min at pH 9.0 (adjusted using 1 N NaOH). Reaction was monitored using PEI-Cellulose TLC strips (E-Merck, Germany). The mobile phase used was 1.0 M NaH_2PO_4 (pH 3.5). The autoradiograph (Fig. 1) of the TLC strips was obtained.