A RAPID AND IMPROVED METHOD FOR THE SYNTHESIS OF ^{32}P-LABELLED RIBONUCLEOSIDE 5'-MONOPHOSPHATES*

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A method, modified from those of Tener (1961, 1962) and Haruna, Nozu, Ohtaka and Spiegelman (1963) has recently been described for the small-scale preparation of ^{32}P-labelled ribonucleoside 5'-monophosphates (Greenlees and Symons, 1966). This method involved the condensation of orthophosphoric acid, 2-cyanoethanol and isopropylidene nucleoside in anhydrous pyridine with dicyclohexylcarbodiimide as condensing agent. In this paper is described a simpler and more rapid method for the preparation of labelled mononucleotides from orthophosphoric acid and isopropylidene nucleoside in dimethyl formamide using trichloroacetonitrile as the condensing agent (cf. Cramer and Weimann, 1961; Pfitzner and Moffatt, 1964). The reaction is complete in under 15 minutes and, after hydrolysis of the protecting isopropylidene group and purification of the reaction mixture by paper electrophoresis, the mononucleotides can be isolated in 25% to 50% yield relative to phosphate added.

EXPERIMENTAL

Materials: Reagent grade pyridine was dried by distillation from phosphorus pentoxide and storage over calcium hydride. Dimethyl formamide was

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distilled from and stored over calcium hydride. Trichloroacetonitrile (K & K Laboratories Inc.) was distilled before use. The chromatographic solvent A consisted of tert-butanol, methylethylketone, water, formic acid (44:44:11:0.26).

Vacuum line: The method described requires the use of a simple vacuum line, the construction and use of which have been described by Greenlees and Symons (1966). The reaction flasks consisted of a B10 Quickfit socket, a neck 5.0 cm. long and a bulb 3.0 cm. in diameter.

^{32}P-Uridine 5'-monophosphate: ^{32}P-Orthophosphoric acid in dilute HCl (1 mC) and 1.0 µmole of non-radioactive orthophosphoric acid (0.1 ml. of a 10mM aqueous solution) were added to a reaction flask and taken to dryness on a rotary evaporator. The flask was then evacuated with an oil pump on the vacuum line to ensure complete removal of the HCl. Uridine isopropylidene (14.3 mg.; 50 µmoles) was added in pyridine to the flask and the mixture taken to dryness on the vacuum line. Pyridine, from the bulk pyridine supply on the vacuum line, was then twice distilled into and out of the flask to ensure thorough drying of the components. Dry air was let into the vacuum line and the flask removed and stoppered. Dry dimethyl formamide (0.5 ml.) was then added to the flask which was warmed to ensure complete solution of the contents. Next, 0.05 ml. of dry dimethyl formamide containing 10 µmoles trichloro acetonitrile (prepared by making 0.020 ml. trichloroacetonitrile to 1.0 ml. with dry dimethyl formamide) was added to the flask, the contents well mixed and incubated at 37^{o} for 20 minutes.

At the completion of the reaction, the flask was taken to dryness on the vacuum line, 1.0 ml. 2N acetic acid added and the flask heated for one hour at 100^{o} to remove the protecting isopropylidene group. After removal of the acetic acid by twice taking the flask to dryness on a rotary evaporator, the contents were dissolved in one ml. of water and fractionated as a band 30 cm. long by low voltage paper electrophoresis in 0.025M sodium citrate, pH 5.0, as described by Greenlees and Symons (1966) using the apparatus of Rushizky and

Knight (1960). Potential gradients of 9 volts/cm. for 5 hours or 4 volts/cm. overnight were sufficient to ensure complete separation of the mononucleotide from the faster moving inorganic phosphates and the small amounts of P^1, P^2-diuridine pyrophosphate and UDP which were also formed during the reaction. The mononucleotide band was cut out and eluted with 0.1mM EDTA, pH 7.0, and the eluted material stored at -15° in the same buffer. Whenever removal of the citrate buffer prior to elution was required, the electrophereogram was cut just behind the mononucleotide band and the paper subjected to descending chromatography overnight in Solvent A; in this solvent the citrate migrates but not the nucleotides (Fink, Cline and Fink, 1963).

The method described provided labelled UMP with a specific activity of one mC/μmole in a yield of about 45% relative to the labelled phosphate added (Table 1).

^{32}P-Adenosine, cytidine and guanosine 5'-monophosphate: For the preparation of these nucleotides, the method as described for UMP was followed except that the reaction mixtures were as given in Table 1, together with the yields obtained.

DISCUSSION

The method described for the synthesis of the ^{32}P-labelled ribonucleoside 5'-monophosphates has been found to be simple, rapid and reliable. Since only one μmole of orthophosphoric acid is required in each reaction mixture, the method allows the synthesis of nucleotides of high specific activity.

The proportion of the components of the reaction mixtures was found to have a marked influence on the nucleotide yield, especially for the synthesis of CMP and GMP. For example, with 1.0 μmole phosphoric acid, 50 μmoles isopropylidene cytidine or guanosine and 10 μmoles trichloroacetonitrile (i.e. as for UMP, Table 1), yields were down to 1%. On the other hand, for the synthesis of AMP, variation of the nucleoside from 5 to 50 μmoles and trichloroacetonitrile from 5 to 25 μmoles had little effect on the nucleotide

TABLE 1.

REACTION MIXTURES FOR PREPARATION OF LABELLED NUCLEOTIDES*

Nucleotide	Reaction components (μmoles)			Yield** %
	Phosphoric Acid	Isopropylidene Nucleoside	Trichloro-acetonitrile	
AMP	1.0	10	25	35 - 45
CMP	1.0	10	25	26 - 32
GMP	1.0	10	25	35 - 45
UMP	1.0	50	10	40 - 50

*Reaction carried out at 37° for 20 minutes under anhydrous conditions in 0.55 ml. dimethyl formamide.

**Relative to labelled orthophosphoric acid added.

yield. Likewise, yields of UMP have not varied as much but highest yields have been obtained with relatively high amounts of the nucleoside and low amounts of trichloroacetonitrile (Table 1).

The method described should be directly applicable to the synthesis of labelled deoxymononucleotides using the suitably protected nucleoside in reaction mixtures known to give maximum yields. So far, deoxythymidine 5'-monophosphate has been obtained in 30% yield using 3'-O-acetyl thymidine and the amounts of components in the reaction mixture as described for AMP, CMP and GMP in Table 1. When the reaction mixture of UMP was tried, yields of dTMP were less than 1%.

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