# AN ENZYMATIC PROCEDURE FOR THE SYNTHESIS OF $^{3}H$ -THYMIDINE-5'-TRIPHOSPHATE

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## SUMMARY

An enzymatic method for synthesis of tritium-labelled thymidine-5'-triphosphate is described. The procedure utilizes a nucleoside phosphotransferase from wheat germ and a partially purified thymidylate kinase from calf thymus. The product is isolated by ion-exchange chromatography on DEAE-cellulose and can be obtained in a yield of approximately 40% and at a radiochemical purity of 95%.

Key Words: Thymidine-5'-triphosphate, Tritium, Nucleoside phosphotransferase, Thymidylate kinase, DEAE-cellulose

## INTRODUCTION

In this communication we want to report on a new method for enzymatic synthesis of tritium-labelled TTP* on a preparative

*Abbreviations: TdR, thymidine; TdR-5'-F, 5'-deoxy-5'-fluoro-thymidine; TMP, TDP, and TTP, thymidine-5'-mono-, di-, and triphosphate; ATP, adenosine-5'-triphosphate; IMP, inosine-5'-monophosphate; AMP, adenosine-5'-monophosphate; UMP, uridine-5'-monophosphate; CMP, cytidine-5'-monophosphate; dGMP, deoxyguanosine-5'-monophosphate; 3'F-TMP, 3'-deoxy-3'-fluoro-thymidine-5'-monophosphate

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scale. This compound, a direct precursor of DNA synthesis, is of great interest for biochemical investigations concerning the field of DNA replication, recently in particular as substrate for the RNA-dependent DNA-polymerase of oncogenic viruses.

In comparison with procedures of chemical synthesis of TTP resulting in good yields but requiring relatively high amounts of the starting compound (1), enzymatic methods in general have the advantage of involving practically carrier-free radioactive compounds. The hitherto described enzymatic methods for TTP synthesis using, respectively, a purified enzyme from E. coli (2) and a tryptophan-stabilized enzyme system from regenerating rat liver (3) need high amounts of ATP which are difficult to separate from TTP and result in about 20% yield. Another possibility is to combine the two procedures, first synthesizing the deoxynucleoside monophosphate by a chemical method followed by further phosphorylation to the corresponding nucleoside triphosphate as described by Symmons (4), which gives a yield of 25-50%.

By our method, TdR is phosphorylated to the monophosphate with IMP as the phosphate donor by means of a nucleoside phosphotransferase from wheat germ. For the next phosphorylation step we used a partially purified thymidylate kinase from calf thymus, working with only a threefold excess of ATP over the substrate TMP. These optimal conditions make it possible to apply DEAE-cellulose column chromatography as the single isolation step.

## MATERIALS AND METHODS

Thymidine-methyl-$^{3}H$ (10-21 Ci/mmole) was obtained from the Nuclear Research Institute, Rossendorf. TMP, TDP and TTP were products of Boehringer, Mannheim. ATP and IMP were purchased from Reanal. TdR-5'-F was synthesized in our department. DEAE-cellulose was a product of Serva.

Preparation of wheat seedling nucleoside phosphotransferase

The seeds were sprouted by scattering them on filter paper and kept moist with tap water. They were allowed to germinate at room temperature in the dark for 5-6 days. Then the seedlings were frozen in dry-ice/methanol, ground with a pestle in a chilled mortar in 3 volumes of 5 mM Tris-HCl buffer, pH 8.0, containing 1.2 mM mercaptoethanol and the obtained homogenates were spun down (24000 g, 20 min, 0°C). The supernatant fraction was brought to 90% saturation with ammonium sulfate and the resulting precipitate was centrifuged (27000 g, 20 min, -2°C) and dissolved in 5 mM Tris-HCl buffer, pH 8.0; finally the ammonium salt was removed by passing the solution through a Sephadex G25 column. The fractions were checked for enzymatic activity and the most active fractions were pooled and taken as the source of enzyme.

Preparation of thymidylate kinase from calf thymus

Enzyme purification was performed as described by Kielly (5). The dialyzed solution obtained after step 5 was used as the enzyme. Since thymidylate kinase is rapidly inactivated during incubation in the absence of substrate (6), we supplemented the used buffer solutions with 0.01 mM TdR-5'-F for stabilization of the enzyme (7).

Paper chromatography

The compounds to be checked were separated together with standards by paper chromatography in descending direction on paper strips (Schleicher & Schüll, 2043 b Mgl) with the system acetone--n-butanol--formic acid--5% ammonium formate (5:7:3:5) for 17 hrs at room temperature. The spots were located by ultraviolet light, eluted with water and their radioactivity assayed in a Packard 4322 Tricarb Liquid Scintillation Spectrometer.

Incubation and Reaction Mixtures

Incubation was carried out at 37°C. The mixture for synthesis of TMP by nucleoside phosphotransferase contained 80 mM Tris-HCl buffer, pH 8.0; 0,6 mM $^{3}$H-TdR, 30 mM IMP, 24 mM phosphoglycerate, 10 mM KF and wheat seedling enzyme (0.5 vol. of the whole mixture). The reaction was stopped after 60 min by immersing the tubes in boiling water for 3 min. The centrifuged supernatant containing the newly formed TMP was incubated with thymidylate kinase for further phosphorylation as follows: Per 1 ml supernatant a reaction mixture was added containing 64 mM Tris-HCl buffer, pH 8.0; 24 mM phosphoglyceric acid; 1.5 mM ATP; 5 mM $MgCl_2$ and 0.2 ml partially purified thymidylate kinase (final volume 1.5 ml).

Chromatography on DEAE-cellulose

For separation of the reaction products a column (1.5x38 cm) of DEAE-cellulose was used, prepared by the method of Staehelin (8) and a gradient elution was carried out. The mixing vessel contained 1.5 l twice-distilled water, the reservoir contained 1.5 l of 0.35 M triethylammonium hydrogen carbonate buffer, pH 7.5. The eluates were collected (15 ml per tube) at a flow rate of 45 ml/hr.

## RESULTS AND DISCUSSION

In the beginning of our experiments we used an unpurified extract (100000 g supernatant) from Ehrlich ascites carcinoma cells, which show a high level of thymidine kinase. Since the synthesis of TMP depends on the amount of ATP (4) at first we applied a hundredfold excess of ATP over the substrate. Thus, it is possible to obtain TMP in a yield of 80-90%. But under these conditions we failed to separate the final product TTP, obtained after further phosphorylation by means of thymidylate kinase, from this high excess of ATP

by simple chromatographic procedures. For this reason, we looked for another way for the phosphorylation of TdR avoiding the high amounts of ATP. As it is known from the literature, many plant tissues contain a nucleoside phosphotransferase catalysing the reversible transfer of ester phosphate from nucleoside-5'-monophosphates to a nucleoside and in some cases this enzyme was used for the synthesis of mononucleotides on a small preparative scale (9-11).

We checked this system with TdR and some nucleoside-5'-monophosphates as the phosphate donor, and obtained the results shown in Table I.

Table I. Phosphorylation of TdR by nucleoside phosphotransferase from wheat seedlings

| substrate (0.6 mM) | phosphate donor (30 mM) | % yield of TMP |
|---|---|---|
| TdR | AMP | 66 |
| | TMP | 58 |
| | 3'F-TMP | 54 |
| | IMP | 55 |
| | UMP | 51 |
| | dGMP | 37 |
| | CMP | 29 |

Since the highest rate of phosphorylation was reached with AMP this compound was selected for further experiments with thymidylate kinase to prepare TTP.

To simplify the procedure, the mixture needed for the thymidylate kinase reaction was added after the optimal incubation time of 30 to 60 min used for the nucleoside phosphotransferase step and the incubation was continued for 1 hr. Unexpectedly, we obtained only

a very low yield of TTP in these experiments. Therefore, we checked the influence of AMP on the thymidylate kinase reaction. As we have found, AMP strongly inhibits this reaction; for example, in the presence of 10 mM AMP the phosphorylation of TMP (0.12 mM) by ATP (1.2 mM) shows an inhibition of 64%. As far as we know, the inhibition of the thymidylate kinase reaction by AMP is a new result, not described in the literature hitherto.

For the following experiments we therefore used IMP as a phosphate donor of the nucleoside phosphotransferase reaction, since it gives an average phosphorylation of TdR in the range of 50-60% after 30 min incubation, it proved to be no inhibitor of the thymidylate kinase and is easy to separate by DEAE-cellulose column chromatography from the final product, TTP. The time course of this reaction is shown in figure 1:

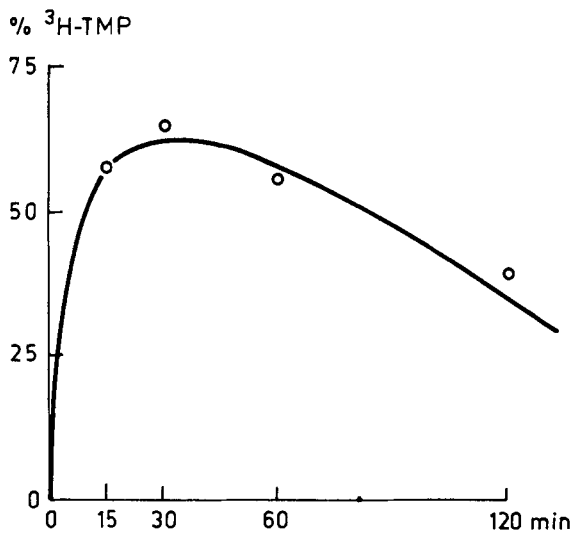


Fig. 1. Time course of $^{3}$H-TdR phosphorylation with IMP by nucleoside phosphotransferase from wheat seedlings. Reaction conditions as described under Methods.

It should be emphasized that the synthesis of TTP by the thymidylate kinase reaction is nearly quantitative using only a 3-fold excess of ATP over the substrate TMP, so that the originally existing problem of separating excess ATP from TTP could be eliminated. The highest yield of TTP is obtained after 1 hr of incubation as can be seen from figure 2:

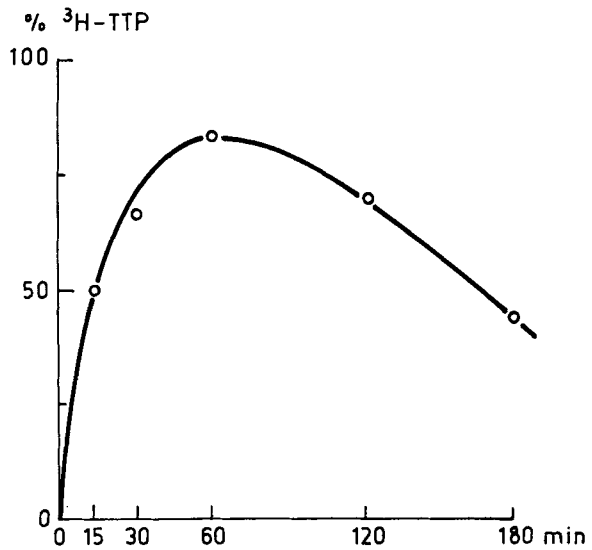


Fig. 2. Time course of phosphorylation of $^{3}$H-TMP to $^{3}$H-TTP by thymidylate kinase of calf thymus. Reaction conditions as described under Methods.

After this time the heat-treated reaction mixture supernatant was concentrated to about 1 ml and applied to the DEAE-cellulose column. Gradient elution was performed as described under Methods. TTP was eluted at approximately 0.15 M buffer concentration in a volume of about 300 ml. The fractions were checked for radioactivity and analyzed by paper chromatography. Thereafter, the samples with the highest content of TTP were pooled and concentrated in vacuo.

In this way, we obtained from some experiments final products with a specific activity in the range of 10-20 Ci/mmole (in dependence on the $^{3}$H-TdR charge) and an average distribution of radioactivity from 95% TTP and 5% TDP. The solutions were stored at -15$^{o}$C in 50% ethanol and were stable without detectable decomposition for at least 3 months.

Summarizing the results, we obtain a phosphorylation of $^{3}$H-TdR to $^{3}$H-TMP from 50-60% by the nucleoside phosphotransferase reaction and a further synthesis of $^{3}$H-TTP in the range of 75-85% by means of thymidylate kinase of calf thymus. The special advantage of the described procedure consists in the use of extremely low amounts of ATP. Thus, under optimal conditions it is possible to obtain $^{3}$H-TTP in a final yield of about 40% using a single purification step.

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