Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXIX, No. 10

ENZYMATIC SYNTHESIS OF ISOTOPICALLY LABELLED PYRIMIDINE DEOXYRIBONUCLEOTIDES.

Frank-Kamenetskaya M.D., Myasoedov N.F.
Institute of Molecular Genetics, USSR Academy of Sciences,
Kurchatov Sq., Moscow 123182

SUMMARY

An enzymatic method is described which makes it possible to synthesize labelled pyrimidine deoxyribonucleotides from corresponding labelled ribonucleotides using the RDP-reductase system of ether-treated E. coli E125 cells. Ether-treated cells have been used for the first time for preparative enzymatic synthesis of labelled biologically active compounds.

INTRODUCTION

Enzymatic synthesis provides an unique tool for the production of complex biologically active compounds with several labelled atoms from simple labelled substrates. Methods are known for obtaining highly labelled ribonucleotides from labelled nucleic acid bases and labelled D-ribose using enzymes from the purine and pyrimidine salvage pathways [1,2,3]. Deoxyribonucleotides are synthesized in microorganisms by a direct reduction of the corresponding ribonucleotides [4,5]. The reaction is catalyzed by different enzymes: in E. coli by RDP-reductase (EC 1.17.4.1) [6,7]

0362–4803/91/101131–10$05.00
© 1991 by John Wiley & Sons, Ltd.
Received 1 July, 1991

and in some other microorganisms by RTP-reductase (EC 1.17.4.2) [5]. Ribonucleoside diphosphate serves as a substrate for RDP-reductase and ribonucleoside triphosphate for RTP-reductase. The synthesis of labelled dATP from labelled ATP using RTP-reductase from Lactobacillus leichmannii was described [3]. However, the procedures of the isolation of both these reductases are laborious and time consuming, their activity in crude extracts and partially purified preparations is low [8,4,5]. The treatment of cells by ether makes them permeable to nucleotides and preserves some intracellular structural features needed for optimal activity of the RDP-reductase system [9,10]. The paper describes the enzymatic synthesis of labelled pyrimidine deoxyribonucleotides from the corresponding labelled ribonucleotides using RDP-reductase system of ether-treated E. coli cells.

MATERIALS AND METHODS

Tritium-labelled /1',5,5',6-^{3}H/CDP, -CTP, -UDP, -UTP (70-80 Ci/mM) and tritium- and carbon-14-labelled /5,6-^{3}H,U'-^{14}C/CTP, -UTP (48 Ci/mM) were obtained from simple labelled precursors using the polyenzymatic preparation from E. coli B [2].

TTP and dithiothreitol were purchased from Serva. Creatine phosphate and creatine phosphokinase (2.7.3.2) were from Reanal, apyrase (3.6.1.5) and alkaline phosphatase (3.1.3.1) - from Sigma.

E. coli E125 strain with increased RDP-reductase activity was a generous gift of Prof. J.A. Fuchs [11]. E. coli E125 cells were treated with ether as described by Vosberg and Hoffman-Berling [10].

Labelled cytosine and uracil deoxynucleotides were synthesized at 37^{0}C in a reaction mixture, which consisted of 0.04 M Tris-HCl, pH 8.4, 10 mM $MgCl_2$ and 0.12 ml/ml (2.4 mg protein/ml) ether-treated cells. Saturating concentrations of CDP and UDP - 0.4×10^{-3}M were used (as reported [9,11], K_m for both substrates are

nearly 0.1×10^{-3}M using ether-treated cells). The reducing power for the reaction was supplied by a 10-fold molar excess of dithiothreitol to corresponding ribonucleotide. The cell debris was removed by centrifugation, the supernatant was placed in boiling H_2O for 3 min and protein was removed by centrifugation. In the preparative-scale synthesis an additional filtration with CF25 or CF50 Amicon filters was performed to remove even trace amounts of proteins from the resultant supernatant.

The conversion of ribonucleotide to the corresponding deoxyanalogue was monitored by analyzing samples of the reaction mixture with apyrase. After enzymatic hydrolysis with apyrase, synthesized deoxyribonucleoside monophosphate was separated from corresponding ribonucleoside monophosphate on PEI-cellulose TLC plates (Merk) developed with 0.15 M LiCl saturated with boric acid and neutralized with NH_4OH. This assay, which is described above, measures the sum of all deoxyribonucleoside phosphates formed from corresponding ribonucleoside phosphate and does not distinguish between the various deoxyribonucleoside phosphates, since deoxyribonucleoside tri- and diphosphates are broken down by hydrolysis to monophosphates. The degree of phosphorylation of the sum of ribo- and deoxyribonucleotides was determined by developing PEI-cellulose TLC plates with 1 M NaCl.

The distribution of radioactivity over TLC plates was determined by a BERTHOLD LB 2832 radio-TLC analyzer. Radioactivity was measured by a scintillation counter with 30% registration efficiency in dioxane scintillator.

Purification of the labelled deoxyribonucleotide was performed using ion-exchange DEAE-cellulose-HCO_3^- chromatography (linear gradient 0.05 - 0.3 M triethylammonium bicarbonate, pH 8.6). The synthesized preparations were analyzed by HPLC on a CHROM-5 chromatograph with a Separon SGX C_{18} column eluted with 2% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.0.

RESULTS AND DISCUSSION

ENZYMATIC SYNTHESIS OF DEOXYCYTIDINE PHOSPHATES. We have found that ether-treated E. coli E125 cells exhibit high dephosphorylating activity. This fact has not been reported in literature. Only 40% of cytidine phosphates were present as CDP after 30 min. of incubation, the others degraded to CMP and cytidine. The decrease of ether-treated cells concentration led to the decrease of the dephosphorylation rate, but the rate of reduction also dropped. It was not possible to exceed the 40% conversion of CDP-to-dCMP reduction.

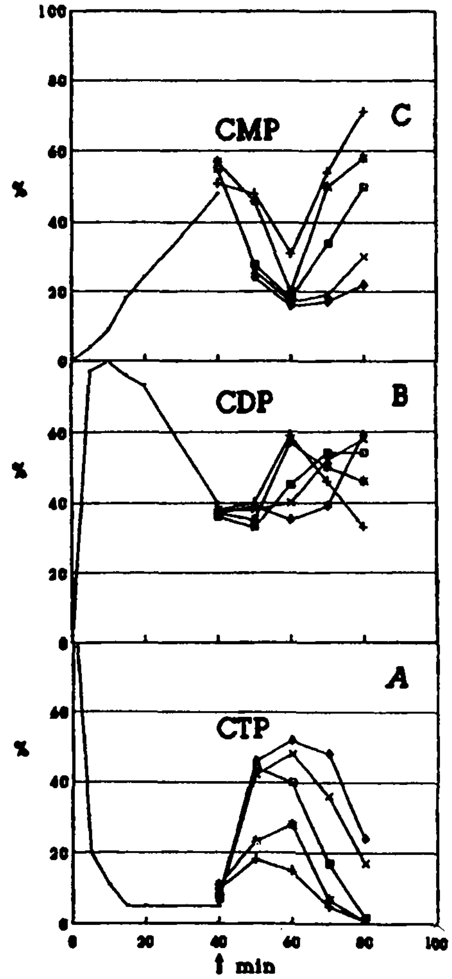

Fig.1 Time course of CTP (A), CDP (B) and CMP (C) content before (—) and after the addition of creatine phosphate and creatine phosphokinase. The molar ratio creatine phosphate/CTP was varied as follows: 1/1 (-+-); 2/1 (-•-); 3/1 (-•-); 4/1 (-*-) and 5/1 (-+-). The arrow indicates the time of the creatine phosphate addition.

After substitution of CDP for CTP, the rapid breakdown of CTP to CDP and CMP occurred and after 20 min of incubation the CTP pool was completely exhausted (Fig.1). Meanwhile, the CDP content remained on the high level of 70-80% between 5 and 20 min of incubation. CTP was therefore chosen as a substrate. The time course for the amount of dCMP formed proved to be very close with the time course with CDP as a substrate, except that the amount of cytidine formed did not exceed 10%. The reduction stopped apparently as a result of the CTP-to-CMP breakdown. It was,

therefore, decided to add to the reaction mixture, which contained CMP-kinase and different dephosphorylating enzymes of ether-treated cells, an ATP-regenerating system, such as creatine phosphate + kinase. We hoped that this might result in the increase of the diphosphate level of cytidine phosphates. Fig.1 shows the time curve of cytidine mono-, di- and triphosphates content during 40 min of incubation and after addition at that moment of different quantities of creatine phosphate + kinase. We concluded that the maximum increase in CDP content occurs within 20 min after the addition of the equimolar creatine phosphate/ATP ratio.

Three such portions of creatine phosphate were added to the reaction mixture every 20 min to maintain a high CDP level during incubation time. As a result the CDP+dCDP content was kept on the level of 60-80% of the initial CTP content. After 80 min the yield of deoxycytidine phosphates reached 85% (Fig.2). Fig.3 shows that it is mostly dCDP. Incubation for extra 30 min with creatine phosphate/CTP ratio of 25:1 led to a 80% yield of dCTP (Fig.3). dCMP and deoxycytidine were obtained with the 90% yield by

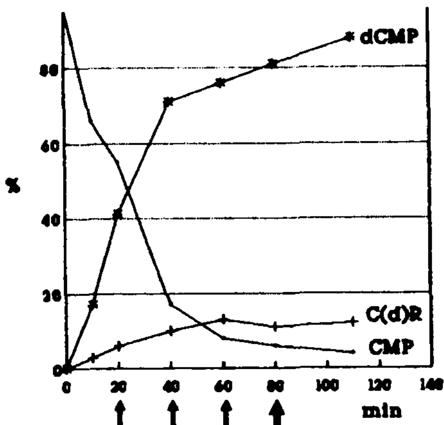

Fig.2 Time course of dCMP (-*-), (deoxy)cytidine (-+-) and CMP (—) content after dephosphorylation using apyrase. Arrows indicate instants of time of the creatine phosphate addition.

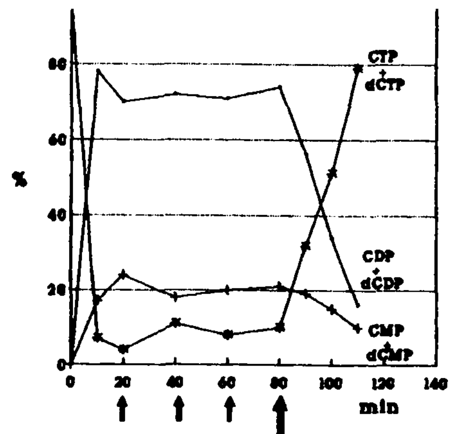

Fig.3 Time course of CMP+dCMP (-+-), CDP+dCDP (—) and CTP+dCTP (-*-) content. Arrows indicate instants of time of the creatine phosphate addition.

enzymatic hydrolysis of dCDP or dCTP with apyrase and alkaline phosphatase respectively.

The reducing power of the reaction can be supplied by dithiothreitol. It was shown that the yield of deoxycytidine phosphates levelled off at the dithiothreitol/CTP ratio of about 10:1.

RDP-reductase is allosterically controlled by deoxynucleoside triphosphates and ATP: ATP and TTP serve as positive effectors, and dATP serves as a negative effector [6,9]. Thus, ATP stimulates activity optimally at 10^{-4}M in ether-treated cells [9]. We, however, did not observe any increase in the rate of CDP reduction in the presence of ATP, probably, due to a high dephosphorylating activity of ether-treated E. coly E125 cells. In addition, possible reduction of ATP may result in a strong inhibition of RDP-reductase by deoxyadenosine phosphates formed. We did not examine TTP because of its high price and nearly complete conversion of CTP into its deoxyanalogue even in the absence of the effector.

Fig.4 shows the preparative run of d/1',5,5',6-^{3}H/CTP (70 Ci/mM, 8.2 μM) synthesized from /1',5,5',6-^{3}H/CTP (72 Ci/mM, 10 μM).

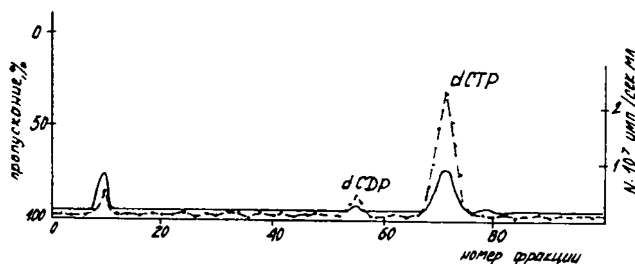

Fig.4 The preparative DEAE-cellulose run of the CTP-to-dCTP reduction products (---- radioactivity, —— optical density).

Retention times of CTP and dCTP in our chromatographic conditions were equal, but analysis of fractions 66-78 by HPLC indicated that only dCTP was contained in these fractions. Thus, DEAE-cellulose chromatography alone is sufficient to isolate the synthesized product due to the complete CTP-to-dCTP conversion.

ENZYMATIC SYNTHESIS OF DEOXYURIDINE PHOSPHATES. Experiments on the uridine phosphate reduction were carried out taking into account the results obtained on the reduction of cytidine phosphates. UTP was used as a substrate, and in the absence of positive effectors the yield of deoxyuridine phosphates did not exceed 40%. An optimal stimulation of UDP reduction with purified proteins of RDP-reductase was reported at concentrations of ATP - 10^{-3}M and TTP - 10^{-4}M [6]. For the reasons mentioned above, ATP was not used as an effector. Addition of 10^{-4}M TTP resulted in some increase (up to 60% of the yield of deoxyuridine phosphates), while further increase of the TTP concentration up to 10^{-3}M resulted in decrease of the yield down to 20%. It is difficult to give an unambigious interpretation of these data because the addition of TTP influences numerous enzymatic transformations. For example, the UDP-to-UTP phosphorylation by nucleoside diphosphate kinase of ether-treated E. coli E125 cells may be responsible for the decrease of deoxyuridine phosphates yield with increasing TTP. Moreover, TTP as well as UTP, serves as a substrate for dephosphorylating enzymes of ether-treated E. coli E125 cells. This may decrease the rate of the UTP-to-UDP breakdown, as we have seen in our experiments. The obtained data indicate that nearly 75% of TTP degrades to TDP and TMP within the first 20 min. Thus, the stimulating action of TTP manifests itself only at the beginning of the incubation. Nevertheless, the addition of portions of TTP during incubation did not increase the yield of deoxyuridine phosphates.

Our study of the UTP dephosphorylation in ether-treated E. coli E125 cells in the presence of 10^{-4}M TTP showed that it differs significantly from the CTP dephosphorylation. Fig.5 indicates that UTP degrades mainly to UDP. The UTP-to-UDP dephosphorylation levels off at about 70-80% after 20 min of incubation, while the UMP level does not exceed 10%. So one can use UDP, as well as UTP,

as a substrate due to the absence of UDP dephosphorylating enzymes in ether-treated E. coli E125 cells.

Fig.6 shows time courses of the deoxyuridine phosphates interconversion while UTP and different amounts of ether-treated E. coli E125 cells were used. One can see that the increase of the amount of ether-treated cells to more than 0.3 ml(6 mg of

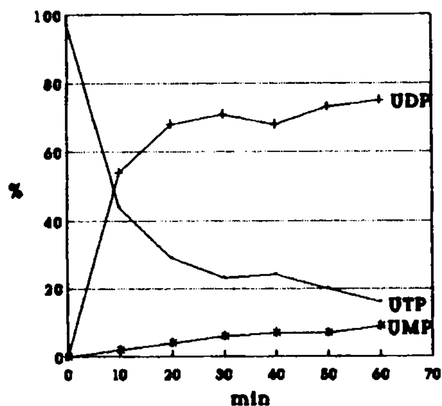

Fig.5 Time course of UTP (—); UDP (-+-) and UMP (-*-) content in the presence of 10^{-4}M TTP.

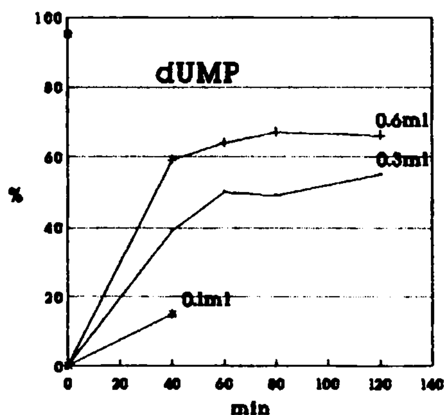

Fig.6 Time course of dUMP formation using 0.1 (-*-), 0.3 (—) and 0.6 (-+-) ml of ether-treated cells in the standard reaction mixture.

protein/μmole UTP) does not result in increased yield of deoxyuridine phosphates. The yield of deoxyuridine phosphates reached 55% after 60 min of incubation. Due to the action of nucleoside diphosphokinase and deoxyuridine triphosphate pyrophosphatase of ether-treated E. coli E125 cells, essentially all of deoxyuridine phosphates produced in these assays were recovered as dUMP. We did not succeed in obtaining dUDP and dUTP after the addition of ATP, ATP-regenerating system and the polyenzymatic preparation from E. coli [2], apparently, because of a low dUMP-kinase activity in E. coli. Deoxyuridine was obtained by enzymatic hydrolysis of dUMP using alkaline phosphatase with the 95% yield.

The described method provides an efficient synthesis of multiply labelled deoxycytidine, deoxyuridine and their mono-, di- and triphosphates of high purity. The type of labels, the number of labels and their location in the synthesized molecule are defined by the labelled substrate.

Our goal was to develop a series of preparative enzymatic syntheses of high specific radioactivity deoxycytidine, deoxyuridine and their phosphates multiple labelled with ^{3}H and/or ^{14}C. The results of several preparative syntheses are presented in Table 1.

Table.1 The results of preparative enzymatic syntheses of labelled cytosine and uracil deoxynucleotides and -nucleosides.

Labelled substrate	A_{mol} Ci/mmol	Labelled product	A_{mol} Ci/mmol	Yeild %	RCP* %
/1',5,5',6-^{3}H/CTP	72	d/1',5,5',6-^{3}H/CTP	70	82	97
/5,6-^{3}H,U'-^{14}C/CTP	48	d/5,6-^{3}H,U'-^{14}C/CTP	47	83	98
d/1',5,5',6-^{3}H/CTP	70	d/1',5,5',6-^{3}/CMP	67	94	99
d/1',5,5',6-^{3}H/CMP	67	d/1',5,5',6-^{3}H/CRib	69	9I	97
/1',5,5',6-^{3}H/UTP	81	d/1',5,5',6-^{3}H/UMP	78	48	97

* - radiochemical purity

Table 1 shows that the radioactive yield of the preparations was no less than 48%. Their radiochemical purity was above 97%. The specific radioactivity of the obtained preparations was equal to the specific radioactivity of the substrate. This indicates that the exogenous radioactive (deoxy)ribonucleotides were not deluted by endogenous (deoxy)ribonucleotides of ether-treated cells. We conclude that ether-treated E. coli cells can be used to syhthesize enzymatically a wide variety of labelled nucleic acid precursors.

REFERENCES

1. Pumpen P.,Taurin V.and Gren E.-Biokhimia 39:504(1974)
2. Frank-Kamenetskaya M.D., Knizhnikova G.V. and Myasoyedov N.F.-Bioorg.Khimia 10:515(1984)
3. Parkin D.W., Leung H.B. and Schramm V.L.-J.Biol.Chem.259:9411(1984)
4. Thelander L., Sjoberg B.M. and Ericsson S.- Meth.Enzymol.,New York, Acad.Press 51:227 (1978)
5. Blaklay R.L.- Meth.Enzymol.,New York, Acad.Press 51:246 (1978)
6. Larsson A. and Reichard P.-J.Biol.Chem.241:2533(1966)
7. Larsson A. and Reichard P.-J.Biol.Chem.241:2540(1966)
8. Reichard P.,Baldesten A., and Rutberg L.-J.Biol.Chem.236:1150 (1961)
9. Warner H.R.-J.Bacteriol.115:18(1973)
10. Vosberg H. and Hoffman-Berling H.-J.Mol.Biol.58:739(1971)
11. Fulpula D. and Fuchs J.A.-J.Bacteriol.-130:107(1977)