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ENZYMATIC SYNTHESIS OF ISOTOPICALLY LABELLED PURINE DEOXYRIBONUCLEOTIDES.

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SUMMARY

An enzymatic method is described which makes it possible to synthesize labelled purine deoxyribonucleotides from corresponding labelled purine ribonucleotides using the RDP-reductase system of ether-treated E. coli E125 cells.

INTRODUCTION

Enzymatic synthesis is widely used for the production of complex biologically active compounds with several labelled atoms from simple labelled substrates. Crude extracts, partially and highly purified preparations from different sources are used as enzyme preparations.

We found that ether-treated cells may be succesfully used for the preparative enzymatic synthesis of labelled nucleic acid precursors [1]. RDP-reductase system (EC 1.17.4.1) of ether-treated E.coli cells catalyzes direct reduction of nucleoside diphosphate to corresponding deoxynucleoside diphosphate [2,3]. We showed that tritium- and carbon-14-labelled cytosine and uracil deoxyribonucleotides are synthesized with quantative yields from their labelled riboanalogues by using RDP-reductase system of ether-treated E. coli E125 cells [1]. Ribonucleoside triphosphates

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were used as substrates because of the high dephosphorylating activity of ether-treated E. coli E125 cells.

In the present paper we study enzymatic synthesis of labelled purine deoxyribonucleotides from corresponding labelled purine ribonucleotides using the same RDP-reductase system of ether-treated E. coli E125 cells. The reduction of adenine nucleotides posed special problems, since products of the reaction, dADP and dATP, act as strong inhibitors of RDP reductase [3]. For this reason the preparative-scale adenine deoxynucleotide synthesis with high yield seemed to be hardly probable.

MATERIALS AND METHODS

Tritium-labelled /1',2,5',8-^{3}H/ATP (78 Ci/mmol), /1',5',8-^{3}H/GTP (57 Ci/mmol) and tritium- and carbon-14-labelled /2,8-^{3}H,U'-^{14}C/ATP, (51 Ci/mmol) were obtained from simple labelled precursors using the polyenzymatic preparation from E. coli B [4].

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

E. coli E125 strain with increased RDP-reductase activity was a generous gift of Prof. J.A. Fuchs [5]. E. coli E125 cells were treated with ether as described by Vosberg and Hoffman-Berling [6].

Labelled adenine and guanine 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 0.1 ml/ml (2 mg protein/ml) ether-treated cells. Substrate concentrations were 0.8×10^{-4}M ATP and 5×10^{-4}M GTP. We used such a low ATP concentration to avoid high concentration of the product, dATP, which is a strong inhibitor of RDP-reductase.

The reducing power for the reaction was supplied by a 10- or

50-fold molar excess of dithiothreitol to GTP and ATP respectively.

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 and the degree of phosphorylation of the sum of ribo- and deoxyribonucleotides was determined as described in [1].

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

Isolation and purification of synthesized labelled deoxyribonucleotide was performed using two-step chromatography. At the first step the ion-exchange DEAE-cellulose-HCO_3^- chromatography (linear gradient 0.05 - 0.3 M triethylammonium bicarbonate, pH 8.6) was used to isolate the sum of ribonucleotide + deoxyribonucleotide from the reaction mixture. At the second step the 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 was used to separate deoxyribonucleotide from its riboanalogue.

RESULTS AND DISCUSSION

ENZYMATIC SYNTHESIS OF DEOXYADENOSINE PHOSPHATES. The ether-treated E. coli E125 cells have been found to exhibit much higher ATP dephosphorylating activity than CTP- and UTP dephosphorylating activities. Dephosphorylation occures so fast that in 5-8 min ATP is entirely degraded to ADP, AMP and adenosine. In order to prevent the ADP-to-(AMP+adenosine) breakdown an ATP-regenerating system, such as creatine phosphate + creatine

phosphokinase, was added after 2 min of incubation. An increase in the creatine phosphate/ATP molar ratio to 8/1 resulted in some increase of adenine deoxynucleotide formation. We did not succeed in the increase of the yield by the addition of different creatine phosphate portions in different moments of incubation.

The positive effector of ADP reduction is dGTP. We have shown that the yield of deoxyadenosine phosphates levelled off at the dGTP/ATP molar ratio of 2/1. It is likely that such a high dGTP content is neccesary because of the high dGTP dephosphorylating rate or/and in order to decrease the rate of adenine nucleotides dephosphorylation.

The reducing power of ADP-to-dADP conversion is supplied by dithiothreitol. We have demonstrated that the yield of deoxyadenosine phosphates levelled off at the dithiothreitol/ATP molar ratio of 50/1.

Fig.1 shows that an increase in the ether-treated cells content to more than 1 ml (20 mg of protein) per 1 μmol ATP does not result in increase of deoxyadenosine phosphates yield.

Experiments were done to demonstrate changes in the phosphorylation degree of the sum of adenine nucleotide + adenine deoxynucleotide during incubation. Fig.2 shows the time course for the conversion of the substrate - ATP to ADP+dADP and AMP+dAMP. It is seen that after 10 min of incubation ADP+dADP content is kept on the level of 60% of the initial ATP content. To 30 min ATP is exhausted and 30% of adenine (deoxy)nucleotides are present as monophosphates. Thus, Figs.1 and 2 show that in 50 min the yield of adenine deoxynucleotides reaches 60%, being mostly deoxyadenosine diphosphate.

In order to obtain dATP, the reaction mixture after 50 min of incubation was separated from cells and protein and phosphorylation was performed during exstra 15 min with creatine phosphokinase and creatine phosphate in 25/1 molar ratio with ATP.

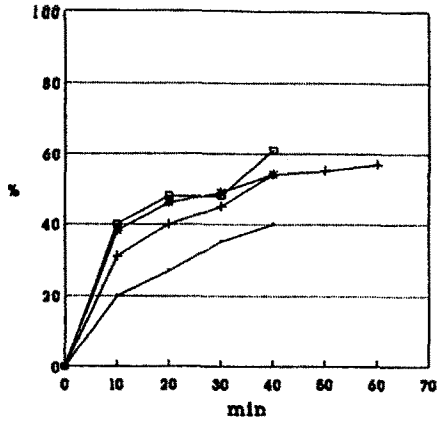

Fig.1 Time course of dAMP formation using 0.5 (—), 1.0 (-+-), 1.5 (-*-) and 3.0 (-o-) ml of ether-treated cells per 1 µmole of ATP in the standard reaction mixture (after dephosphorylation of (deoxy)nucleotides using apyrase).

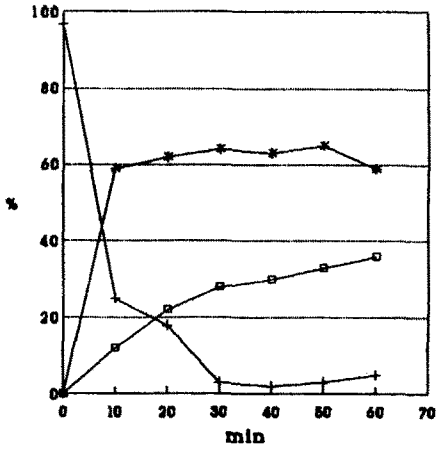

Fig.2 Time course of AMP+dAMP (-o-), ADP+dADP (-*-) and ATP+dATP (-+-) content in the standard reaction mixture.

dAMP and deoxyadenosine were obtained with nearly 90% yield by enzymatic hydrolysis of dADP or dATP with apyrase or alkaline phosphatase respectively.

Fig.3 demonstrates the final HPLC step of dATP isolation, i.e. dATP separation from ATP.

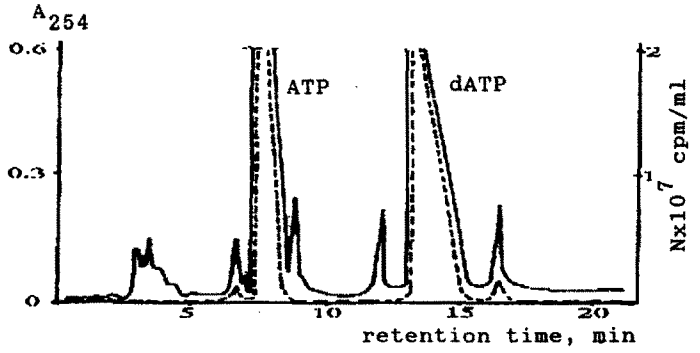

Fig.3 The preparative HPLC Separon SGX C18 run of the ATP-to-dATP reduction products (---- radioactivity, —— optical density).

ENZYMATIC SYNTHESIS OF DEOXYGUANOSINE PHOSPHATES. Experiments on guanine nucleotide reduction were carried out taking into account the results obtained on the reduction of cytosine-, uracil- [1] and adenine nucleotides. GTP was used as a substrate and TTP as a positive effector [3]. GTP-to-(GDP+GMP+guanosine) dephosphorylation was fast and in order to prevent GDP-to-(GMP+guanosine) breakdown after 2 min creatine phosphate in molar ratio with GTP of 8/1 and creatine phosphokinase were added.

The yield of deoxyguanosine phosphates levelled off at the TTP/GTP molar ratio of 0.5/1 and at the dithiothreitol/ATP molar ratio of 10/1.

Fig 4 shows the dependence of the dGMP yield on the amount of ether-treated E. coli E125 cells. One can see that increase in the amount of ether-treated cells to more than 0.2 ml (4 mg of protein) per 1 μmol GTP does not result in increased yield of deoxyguanosine phosphates. The yield of deoxyguanosine phosphates reached 60% after 60 min. It was shown that essentially all of deoxyguanosine phosphates were recovered as dGDP.

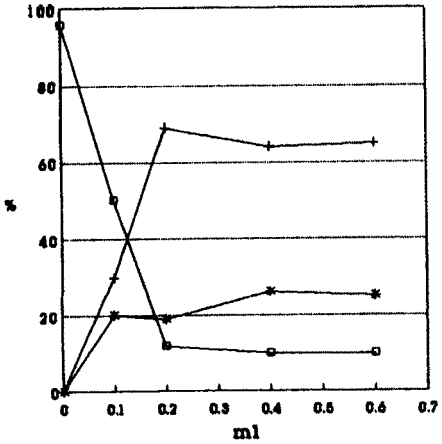

Fig.4 GTP-to-(dGMP+(deoxy)guanosine) conversion after dephosphorylation of (deoxy)nucleotides with apyrase. The dependence of GMP (-o-), dGMP (-+-) and (deoxy)guanosine (-*-) content on the amount of ether-treated E. coli E125 cells.

To obtain dGTP, the reaction mixture was separated from cells and protein after 60 min of incubation and phosphorylation was performed during 15 min with creatine phosphokinase and creatine phosphate in 25/1 molar ratio with GTP.

dGMP and deoxyguanosine were obtained with nearly 90% yield by enzymatic hydrolysis of dGDP or dGTP with apyrase or alkaline phosphatase respectively.

A series of preparative enzymatic syntheses of high specific radioactivity deoxyadenosine, deoxygaunosine and their phosphates multiply labelled with ^3H and/or ^{14}C was carried out. Tabl.1 presents the results of several preparative syntheses.

Table.1 The results of preparative enzymatic syntheses of labelled adenine and guanine deoxynucleotides and -nucleosides.

Labelled substrate	A_{mol} Ci/mmol	Labelled product	A_{mol} Ci/mmol	Yield %	* RCP %
/1',2,5',8-^{3}H/ATP	78	d/1',2,5',8-^{3}H/ATP	74	48	98
/2,8-^{3}H,U'-^{14}C/ATP	51	d/2,8-^{3}H,U'-^{14}C/ATP	48	51	97
d/1',2,5',8-^{3}H/ATP	74	d/1',2,5',8-^{3}/AMP	75	95	99
d/1',2,5',8-^{3}H/AMP	75	d/1',2,5',8-^{3}H/ARib	75	91	97
/1',5',8-^{3}H/GTP	57	d/1',5',8-^{3}H/GTP	59	61	97

* - radiochemical purity

One can see that the radioactive yield of the preparations was not 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.

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