# SYNTHESIS OF $^{14}$C-URIDINE-5′-TRIPHOSPHATE BY ENZYMES OF ESCHERICHIA COLI CELL-FREE EXTRACTS.

P. Pumpen, E. Gren.

Institute of Organic Synthesis,
Acad. Sci. of the Latvian S.S.R.
Riga.

Received on August 6, 1971.

## SUMMARY

*Crude cell-free extracts of Escherichia coli AB 259 Hfr 3 000, thi⁻ perform multistep conversion of $^{14}$C-uracil to $^{14}$C-UTP in vitro in the presence of D-ribose/or ribose-5-phosphate/, ATP and ATP-regenerating system. ATP concentration is crucial for significant $^{14}$C-UTP production. Quantitative yield of the product combined with relatively simple procedure and inexpensiveness of initial materials makes the method advantageous for large-scale preparation of $^{14}$C-UTP.*

## INTRODUCTION

Uracil as well as orotate can serve as precursors for bacterial synthesis of UMP undergoing phosphoribosylation by enzymes UMP-pyrophosphorylase /EC 2.4.2.9; UMP:pyrophosphate-phosphoribosyltransferase/ and orotidine-5′-phosphate pyrophosphorylase /EC 2.4.2.10; orotidine-5′-phosphate:pyrophosphate-phosphoribosyltransferase/, respectively. The bases are phosphoribosylated in the processes by 5-phospho-α,D-ribosyl-1-pyrophosphate preformed in two consecutive reactions from D-ribose.

UMP-pyrophosphorylase activity was demonstrated in vitro previously in enzyme preparations from Brevibacterium ammoniogenes /I/, several Lactobacillus strains /2,3/, and certain strains of E. coli /2,4,5/. However, the activity has been negligible or completely absent in most E. coli strains examined /2/, therefore, no large-scale method for preparation of radioactive uridine 5′-phosphates from $^{14}$C-uracil was proposed hitherto which would have been based on E.coli extracts. Extracts from Lactobacillus were used instead or the metabolic pathway of $^{14}$C-orotate conversion was pursued /2,3,6,7/; unfortunately, a specially prepared substrate /5-phosphoribosyl-I-pyrophosphate/ had to be utilized in order to obtain significant $^{14}$C-UMP yield. Khesin and co-workers /8/ were the first to suggest the possibility of using crude E. coli extracts to convert some amount of $^{14}$C-uracil to $^{14}$C-UTP in the presence of D-ribose, however no quantitative data had been presented. Moderate yield of $^{14}$C-UMP was also obtained by unpurified B.ammoniogenes extracts and ribose-5-phosphate as substrate /I/.

As regards the following stages of the synthesis - UMP conversion into UTP, nucleoside-5′-monophosphate kinase /EC 2.7.4.4; ATP:nucleoside-5′-monophosphate - phosphotransferase/ and nucleoside-5′-diphosphate kinase /EC 2.7.4.6; ATP:nucleoside-5′-diphosphate - phosphotransferase/ activity of E.coli extracts permits this transformations in quantitative yields /7,9,IO/.

The present report proposes a simple and nearly quantitative method for large-scale preparation of $^{14}$C-UTP from $^{14}$C-uracil in crude E.coli extracts and D-ribose as precursor of 5-phosphoribosyl-I-pyrophosphate.

MATERIALS.

2-$^{14}$C-uracil /IO mCi/mmole/ and 2-$^{14}$C-orotic acid /I2,8 mCi/mmole/ were from V/O IZOTOP /USSR/. Tris was purchased from the Sigma Chemical Co. /USA/. Nucleotides, ribose-5-phosphate /R5P/, Ba salt; D-ribose /D-rib/; creatine phosphate /CP/, Na salt; phosphoenolpyruvate /PEP/, Ag-Ba salt; creatine phosphokinase /CPK/; pyruvate kinase /PK/, deoxyribonuclease were obtained from Reanal /Hungary/.

Potassium salts of R5P and PEP were prepared by passage of HCl-treated commercial preparate solutions through Dowex 50/$H^+$/ column, and neutralization of eluate with KOH. Sodium salt of ATP was adjusted to pH 7.

METHODS.

Growth of E.coli cells and preparation of cell-free extracts.

The bacteria were grown by aeration on rotatory shaker at 37°C to the middle log-phase /7-8x$10^8$ cells/ml/ in peptone medium containing /per 1/: $NH_4Cl$ /I g/, $KH_2PO_4$ /I,3 g/, $K_2HPO_4$ /IO g/, NaCl /0,5 g/, $MgSO_4$ /0,I g/, thiamin /0,0I g/, glucose /4,0 g/, peptone /IO g/, pH 7,2. Cells were harvested by centrifugation and washed twice with cold 0,0I M Tris-HCl,

0,005 M $MgCl_2$ buffer at pH 7,8. The yield was I,5-2 g /wet weight/ per I l of medium.

Cell-free extracts were prepared by grinding cells with twice their weight of glass beads in a chilled mortar /I-2°/. The resulting paste was suspended in an amount of cold 0,I M Tris-HCl buffer, pH 7,4, containing 0,005 M $MgCl_2$, equivalent to 2 times the original wet weight of cells, deoxyribonuclease was added /I0 μg/g of cells/. The suspension was centrifuged at I0 000 rpm for 20 min. The supernatant was further centrifuged at I00 000xg for 60 min using the no. 50 rotor in the Spinco L2 ultracentrifuge. Protein content of the extracts was determined by the modified method of Lowry /II/ using crystalline bovine albumin /Koch-Light Laboratories, Ltd/ as a reference standard. Rapidly frozen crude extracts of E.coli have been stored at -20° for up to one month without appreciable loss of activity. The precipitate occuring upon thawing after I0-I5 days of storage does not affect the enzyme activity, and may be removed.

The frozen extract was warmed up immediately before using it in a water bath at 50° and kept there for 3 minutes, then rapidly cooled to 2-3° in an ice bath. The precipitate was removed by centrifugation and discarded.

## Determination of $^{14}C$-UTP synthesis.

The synthesis of $^{14}C$-UTP was assayed at 37° in incubation mixtures containing besides E.coli extract labelled precursor /$^{14}C$-uracil or $^{14}C$-orotate/, R5P /or D-rib/, ATP, PEP and PK /or CPK and CP/, $Mg^{2+}$ ions /quantities in "Results"/.

The mixtures were placed in a boiling water bath for 2 min after incubation and then centrifuged. The supernatants were analyzed by paper chromatography and paper electrophoresis. The solvent systems employed by chromatography using FN-II paper /GDR/ were /I/ ethanol - I M ammonium acetate, pH 3,8 - 0,I M EDTA, pH 8,2 /75:29:I, v/v/v; I8 h by the ascending technique/ /I2/, and /2/ isobutyric acid - water - conc. ammonia /66:33:I,5, v/v/v; I8 h, descending//I3/. Electrophoresis on FN-II paper was carried out in 0,05 M citrate buffer, pH 5,for I,5 h/30 V/cm/.

The positions of UV-absorbing reference compounds were determined in UV light. Both the chromatogram and the electrophoregram of each sample were cut into a number of strips, I cm wide. The strips were placed in scintillation vials containing 5 ml of toluene scintillation mixture and counted in the liquid scintillation spectrometer /Packard, Tri Carb, Model 3380/.

The preparative descending chromatography using WI5 paper was carried out in /3/: isobutyric acid - water - conc. ammonia /66:33:5; 24 h, descending/; approximately I0 $OD_{260}$ units per cm of start line were spotted on the chromatogram. After development of the chromatograms, they were dried in a current of cold air for 2-3 h, washed with ether by descending technique at 2-3° during 2 days. Zones of $^{14}$C-UTP were eluated with water, the resulting solution was concentrated in a rotary evaporator at 30°, frozen and stored at -20°.

RESULTS.

Course of $^{14}$C-UTP synthesis by E.coli extracts and general requirements for reaction.

Assays of $^{14}$C-uracil incorporation into $^{14}$C-UTP by E.coli

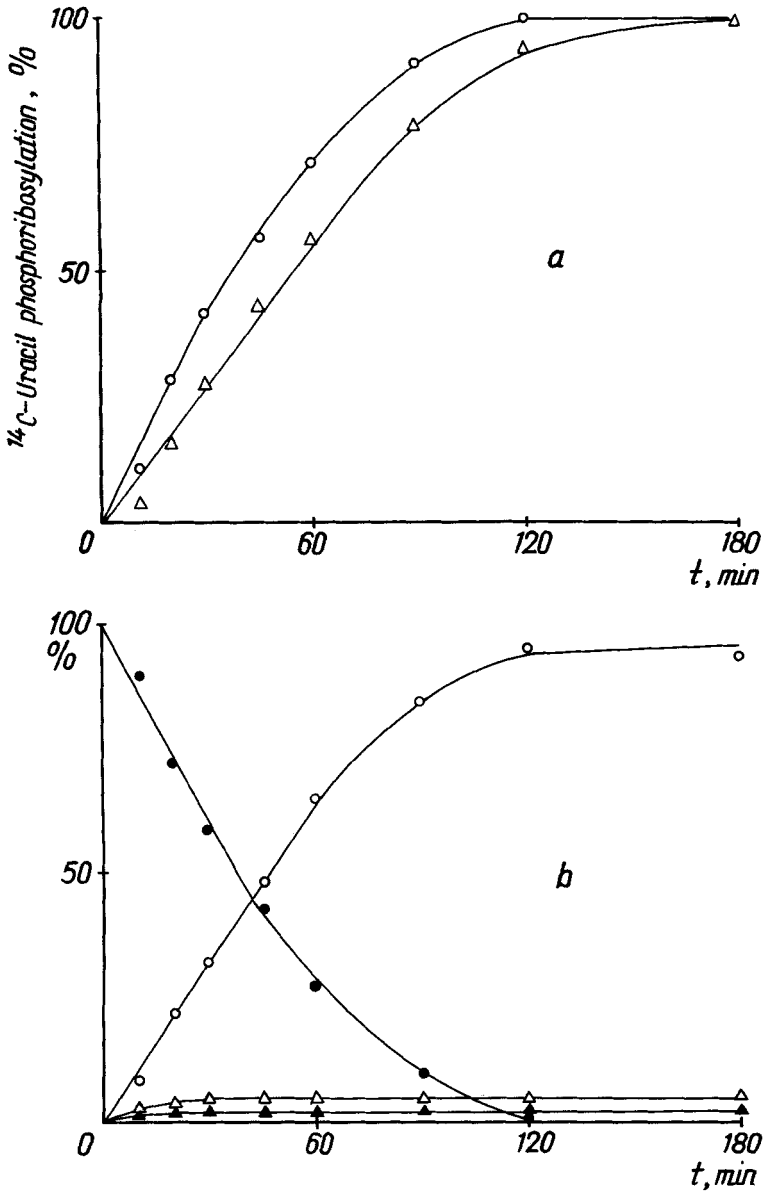
100
50
0
60
120
180
t, min
14C-Uracil phosphoribosylation, %
a
100
%
50
0
60
120
180
t, min
b

Fig. 1.

extracts using both R5P and D-ribose showed that the reaction preserved the relatively high rate for several hours under optimum conditions used /Fig. I/. Amounts of $^{14}$C-uracil and $^{14}$C-uridine nucleotides for various times of incubation are given in Fig. Ib.

The effective synthesis of $^{14}$C-UTP /90-95 % conversion/ proceeded also with $^{14}$C-orotate as precursor under analogous reaction conditions, in accordance with previously reported data /2/ exploiting enzyme preparations from E.coli.

It is evident that the multi-step synthesis of $^{14}$C-UTP by E.coli enzymes proceeded under the ascendancy of different factors affecting the yield of product.

The rate of the resulting process was insignificant as the small amounts of extract were added to the reaction mixture /Fig. 2/. However, the exceeding of optimum enzyme

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Fig. I. Time-course of $^{14}$C-UTP synthesis by E.coli extracts. The reaction mixtures /0,7 ml/ containing /μmole/: Tris-HCl, pH 7,8 - 40; $MgCl_2$ - 15; $^{14}$C-uracil - 1,25; ATP - 1,0; R5P /or D-rib/ - 6,25; PEP - 10; PK - 15 μg; and 0,1 ml of E.coli extract /1 mg of protein/, incubated at 37°.

a/The reaction kinetics using R5P /o-o/ or D-rib /Δ - Δ/.

b/Composition of incubation mixture as function of the incubation time using R5P as substrate. ●-● - $^{14}$C-uracil; o-o - $^{14}$C-UTP; Δ - Δ - $^{14}$C-UDP; ▲-▲ - $^{14}$C-UMP.

as well as $^{14}$C-uracil concentrations led to decrease of $^{14}$C-UTP yield.

Effect of R5P concentration on the resulting reaction is shown in Fig. 3. The optimum rate of synthesis was achieved by using R5P /analogous - D-ribose/ concentration equal fivefold $^{14}$C-uracil concentration.

ATP concentration greater as 2 mM showed an inhibitory effect /Fig. 4/ affecting possibly the 5-phosphoribosyl-I-pyrophosphate formation from R5P /I4/. On the other hand the kinase transformations required the considerable amounts of ATP that forced to compromise. In order to stimulate kinase reactions the ATP concentration may be raised in the final stages of synthesis.

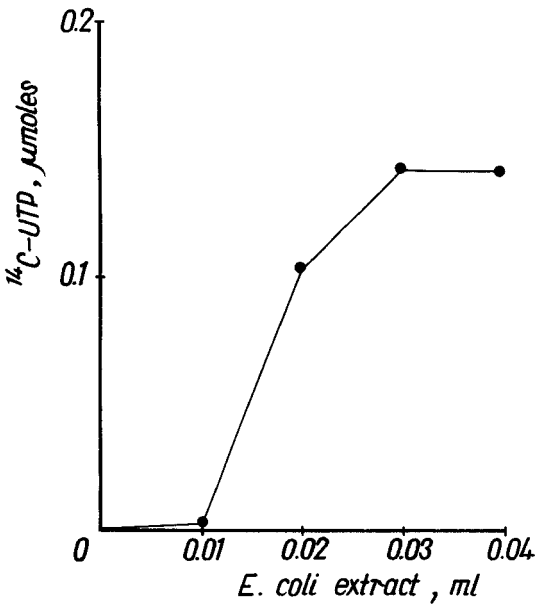


Fig. 2.

Large-scale preparation of $^{14}$C-UTP.

The method is based completely on data presented in previous section "Results". The incubation mixture /30 ml/ containing /mmole/: $^{14}$C-uracil - 0,05; D-rib - 0,25; ATP - 0,045; PEP - 0,2; $MgCl_2$ - 0,75; Tris-HCl, pH 7,8 - 3,0; PK - I mg; E.coli extract - 4 ml /40 mg of proteins/, was incubated for 4 h at 37°. After 3,5 h 0,I mmole ATP, 0,2 mmole PEP and I mg PK were added. The reaction was stopped by immersing the tube in a boiling water bath for 3-4 min. The precipitate was removed by centrifugation. The supernatant solution /after concentration in a rotary evaporator at 30°/ was spotted on WI5 paper for chromatography /system 3/. The yield of $^{14}$C-UTP reached 90-95 per cent. The obtained preparation was radiochemically pure, and had the general UV-absorbance data similar to those for reference compounds.

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Fig. 2. Effect of E.coli enzyme concentration on $^{14}$C-UTP synthesis.
The reaction mixture /0,25 ml/ containing /μmole/: Tris-HCl, pH 7,8 - I2; $MgCl_2$ - 3; ATP - 0,4; $^{14}$C-uracil - 0,2; R5P /or D-rib/ - I,0; PEP - 3,0; PK - 5 μg, and varying amounts of enzyme fraction /I0 mg/ml of protein/ were added. Incubation: I h at 37°.

DISCUSSION.

Cell-free extracts of E.coli are able to convert $^{14}$C-uracil into $^{14}$C-UTP in the presence of D-ribose /or R5P/, ATP and ATP-regenerating system.

The phosphoribosylation of uracil catalized by UMP-pyrophosphorylase seems to be the only possible pathway leading to the synthesis of UTP from uracil which occurs in the bacterial extracts. No evidence supporting the transformation of uracil → uridine → UMP, which occurs in mammalian tissues, is obtained in the present study. These conclusions

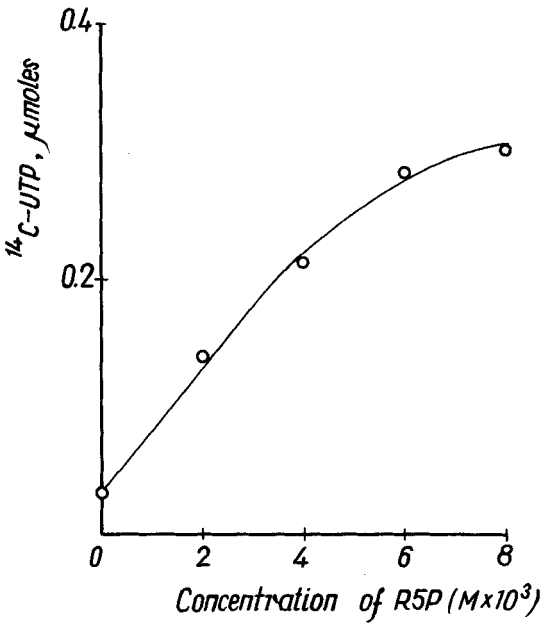


Fig. 3.

coordinate with generally accepted views on enzymatic pathways of uracil metabolism in bacteria /2,3/.

Contrary to literature data /2/, the considerable UMP-pyrophosphorylase activity is demonstrated in cell-free E.coli extracts under the conditions used. An important factor favouring continuous retain of its activity for a comparably long time may be a dynamic equilibrium of all intermediates during the reaction preventing the accumulation of some of them, e.g. UMP, which would inhibit phosphoribosylation of uracil. If to maintain the regeneration of ATP continuously /the chromatographic analysis of samples incubated various time indicates mainly ATP, neither AMP nor ADP/, the equilibria of enzyme reactions using ATP are displaced to the formation of phosphorylated products. Thus UMP already formed is immediately utilized, and its accumulation does not occur. It is of interest to notice that the yield of uridine 5'-phosphates /now consisting of 50 % UTP, 25 % UDP, 25 % UMP in final mixture/ decreases more than tenfold when ATP-regenerating system is omitted. Apparently, the decrease is caused by deficiency of ATP and the accumulation of ADP and AMP, which affects inhibitory all reactions utilizing ATP. Such an explanation is confirmed by the fact that a kind of ATP-regenerating system used /PK + PEP or CPK + CP/ is of little significance.

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Fig. 3. Effect of R5P concentration on $^{I4}$C-UTP synthesis. The reaction mixtures were as given in Fig. 2, except that 0,02 ml of E.coli extract /0,2 mg of protein/ and varying amounts of R5P were added. Incubation: 3 h at 37°.

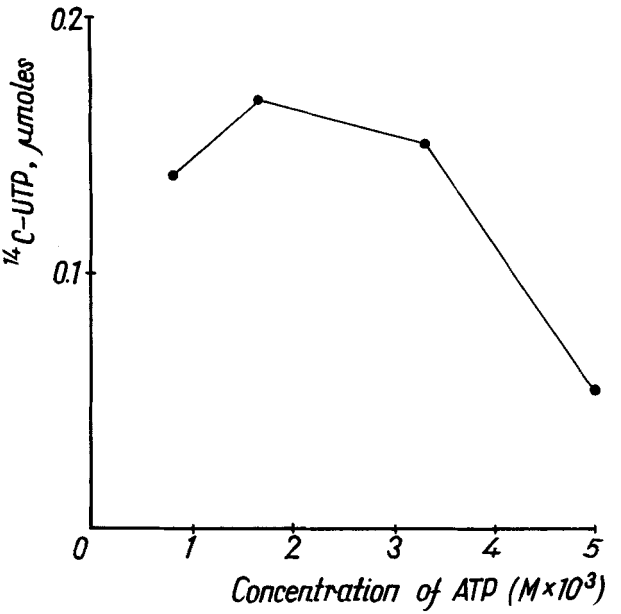


Fig. 4.

Fig. 4. Effect of ATP concentration on $^{14}$C-UTP synthesis. The reaction mixtures were as given in Fig. 2, except that 0,02 ml of E.coli extract /0,2 mg of protein/ and varying amounts of ATP were added. Incubation: 3 h at 37°.

ACKNOWLEDGEMENT.

The authors are indebted to Dr. G. Rosenthal for his interest and valuable advice and Drs. V.R. Bauman and V.N. Markushevich for the culture of E.coli used in this work.

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