NEW POSSIBILITIES OF PREPARATIVE SYNTHESIS OF RADIOACTIVE NUCLEOTIDES
V. ENZYME SYNTHESIS OF TRITIUM LABELLED URIDINE 5'-TRIPHOSPHATE IN HIGH YIELD

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SUMMARY

Growth of Escherichia coli B in the presence of sub-bacteriostatic concentration of various compounds results in the enhancement of activity of enzymes catalyzing the transformation of uridine to its 5'-phosphates. Partially purified extracts of E. coli cultivated in the presence of $1x10^{-5}$M 5-azacytidine were applied for the preparative synthesis of tritium-labelled uridine 5'-triphosphate. Yield of the newly formed nucleotide was about 90% and was higher during the short-term incubation using enzyme extract from the treated cells than that from controls.

INTRODUCTION

The enhancement of enzyme activity in higher organisms and bacteria observed after the administration of various compounds can often be made use of for the technological purposes. We have described the enhancement of the activity

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of thymidine kinase in regenerating rat liver following L-tryptophan[1]. This finding led us to follow optimal conditions for the enzyme synthesis of radioactive thymidine 5′-phosphates[2].

A number of compounds administered to rats results in the increase of hepatic uridine kinase activity[3,4]. The enhancing effect was recently observed also in bacteria. During the cultivation of Escherichia coli B in the presence of low concentration of 5-azacytidine the cells displayed an increased metabolic activity[5]. The effect was limited to enzymes catalyzing the transformation of uridine to corresponding 5′-phosphates.

In this communication the application of partially purified enzyme extract prepared from 5-azacytidine-treated E. coli culture for the synthesis of tritium-labelled uridine 5′-triphosphate is presented. Another method for the synthesis of radioactive 5′-nucleotides by an unpurified cell-free extract of mouse leukemic cells was described by us earlier[6].

MATERIALS AND METHODS

Chemicals. Uridine, uridine 5′-monophosphate, adenosine 5′-triphosphate, creatine phosphate and creatine kinase were obtained from Calbiochem, Luzern. 5-Azacytidine was synthesized in the Institute of Organic Chemistry and Biochemistry, Prague. Uridine-[5-^{3}H] and uridine 5′-monophosphate-[5-^{3}H] were

Abbreviations used: UTP, uridine 5′-triphosphate; UDP, uridine 5′-diphosphate, and UMP, uridine 5′-monophosphate.

prepared in the Institute for Research, Production and Uses of Radioisotopes, Prague. Specific radioactivity of the compounds was 1.0 mCi/μmol.

Preparation of E. coli cell-free extracts. Cultivation of E. coli B was carried out at 37°C for 16 h in 4 litres of a synthetic medium containing glucose[7] with or without 1 x 10^{-5}M 5-azacytidine. For inoculation 100 ml of the 24 hour-old E. coli culture was used. 16 hours later bacteria were centrifuged, washed with 0.9% NaCl and suspended in 40 ml of cold 0.05M Tris-HCl buffer (pH 7.5). The suspension was sonicated (70 sec, 2°C, 1.5 kc) and sonicate centrifuged (10 000 g, 20 min, 2°C). The supernatant fractions used for experiments were stored at -20° C for 4-7 days without detectable decrease of enzyme activity. Partial purification of enzymes present in cell-free extracts was carried out according to modified method of Canellakis[8].

Incubation and analysis of reaction mixture. Incubation was carried out at 37°C in 2 x 10^{-2}M Tris-HCl buffer (pH 8.1) or 2 x 10^{-2}M glycine buffer (pH 7.5) in the presence of E. coli cell-free extract or partially purified enzyme preparation. The detailed composition of incubation mixture is given in Tables. Aliquots of the mixture withdrawn at various time intervals were analyzed by chromatography using Whatman paper No. 3 in a solvent system composed of isobutyric acid--ammonium hydroxide-water (66:1.5:33). Radioactive compounds on chromatograms were located according to standards and by radioactivity scanning. The radioactivity of samples was measured with Packard Liquid Scintillation counter 2212.

RESULTS AND DISCUSSION

The ability of unpurified cell-free extracts prepared from E. coli to metabolize uridine is shown in Table 1. It is apparent that the enzyme extract from the treated culture is more active. After 20 min of incubation uridine is not present in the incubation mixture any more. At the same time significant synthesis of uridine 5′-triphosphate-[5-^{3}H] occurs. Using enzyme extracts from control culture uridine 5′-triphosphate-[5-^{3}H] is not formed at all. The degradation of uridine-[5-^{3}H] to uracil-[5-^{3}H] is relatively high and proceeds in both extracts similarly. The compound designated X was not clearly identified. It is supposed to be related to uridine 5′-diphosphate-[5-^{3}H] with R_F value of 0.40 in the solvent system composed of isobutyric acid-ammonium hydroxide-water (R_F of uridine is 0.55 and that of uridine 5′-monophosphate 0.38).

From the data presented in Table 1 it is evident that unpurified extracts are not convenient for the preparative synthesis of tritium labelled uridine 5′-mono and triphosphate. For this reason both extracts were partially purified to remove contaminating nucleosidases and nucleotidases, and subsequently they were used for the synthesis of tritium labelled uridine 5′-triphosphate. The data are shown in Table 2 and Fig. 1.

The enzyme preparation from 5-azacytidine-treated cells of E. coli is more active. Uridine 5′-monophosphate-[5-^{3}H] is metabolized already during 15 min of incubation period; using control enzyme extract a similar picture was obtained

Table 1. Time Course of Uridine-[5-^{3}H] Conversion in Unpurified Cell-Free Extracts of Control and 5-Azacytidine-Treated E. Coli Culture

Uridine-[5-^{3}H](1 mCi/μmole was incubated with 0.5 ml of E. coli extract at 37°C in a total volume 2.5 ml of 2 x 10^{-2}M Tris-HCl buffer (pH 8.1) with 1 x 10^{-2}M adenosine 5′-triphosphate and equimolar Mg^{2+}-ions. The amount of newly formed compounds is expressed as per cents of the total radioactivity of analyzed sample.

Compound-[^{3}H]	Control enzyme			Stimulated enzyme		
	30 min	60 min	180 min	30 min	60 min	180 min
UTP	0	0	0	32	0	0
UDP	10	8	5	7	13	13
UMP	21	16	14	9	15	21
X	35	37	43	24	38	32
Uridine	9	4	0	0	0	0
Uracil	25	35	38	28	34	35

Table 2. Phosphorylation of Uridine-[5-^{3}H] 5′-Monophosphate to Uridine-[5-^{3}H] 5′-Di and 5′-Triphosphates

Uridine-[5-^{3}H] 5′-monophosphate (1 mCi/5 μmoles) was incubated with 0.3 ml of partially purified enzyme preparations at 37°C with 0.7 ml 2 x 10^{-2}M glycine buffer (pH 7.5), 2.2 x 10^{-3}M adenosine 5′-triphosphate, 2.2 x 10^{-2}M Mg^{2+} -ions, creatine phosphate (8.5 mg) and creatine kinase (0.33 mg). The amount of newly formed compounds is expressed as per cents of total radioactivity of analyzed sample.

Compound-[^{3}H]		Newly formed compounds (%)				
		15 min	30 min	45 min	75 min	120 min
UTP	Control enzyme	36.3	54.9	61.6	80.9	92.1
UDP		34.9	35.1	31.2	14.6	4.2
UMP		18.8	7.5	4.0	0.6	0
UTP	Stimulated enzyme	87.7	91.7	96.0	85.2	80.2
UDP		5.9	2.2	1.8	9.4	8.6
UMP		1.6	0.9	0	0	0

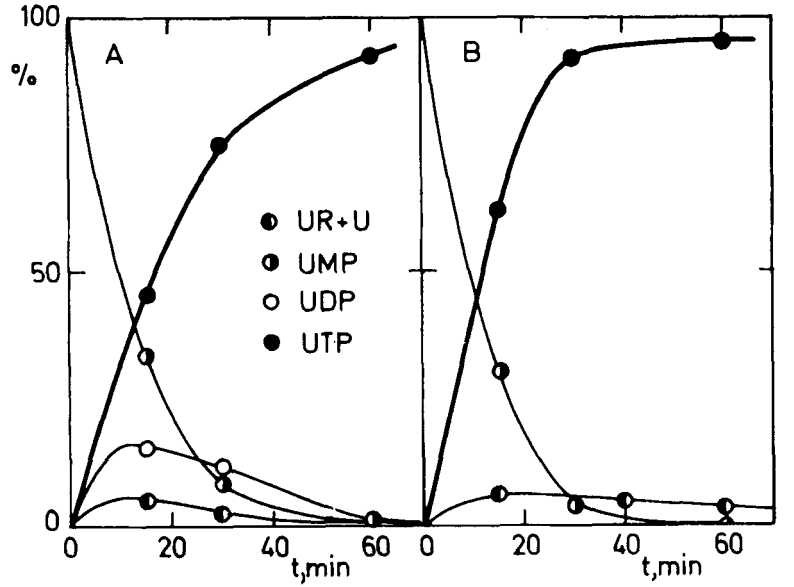

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Figure 1. Time Course of Uridine-5′-Monophosphate-[5-^{3}H] Phosphorylation to Uridine-5′-Triphosphate-[5-^{3}H]

A, Control E. coli culture; B, 5-azacytidine-treated E. coli culture. Incubation of uridine-5′-monophosphate-[5-^{3}H] (1 mCi/5 μmoles) was carried out at 37° C in total volume 1 ml in 2 x 10^{-2}M glycine buffer (pH 7.5) in the presence of 0.1 ml enzyme preparation. Conditions of incubation were the same as in Table 2. The amount of compounds in reaction mixture is expressed as per cent of total radioactivity of analyzed sample.

after 1 hour of incubation (Table 2). Uridine 5′-triphosphate -[5-^{3}H] is formed at a high rate reaching 90-95% yield after 30 min. With the control enzyme preparation the same level of

uridine 5´-triphosphate-[5-^{3}H] was reached only after 2 hours of incubation. However, the results indicate that untreated enzyme extract is more convenient for the synthesis of uridine 5´-diphosphate-[5-^{3}H]. The total radioactivity of all 5´--phosphates formed including the substrate is less than 100%. The remaining percentage is accounted for the traces of uracil--[5-^{3}H], uridine-[5-^{3}H] and by two unidentified compounds. The time course of uridine 5´-triphosphate-[5-^{3}H] synthesis in the presence of the control and stimulated enzyme extract is shown in Figure 1.

The use of partially purified enzyme fraction obtained from E. coli B cultivated in the presence of sub-bacteriostatic concentration of 5-azacytidine for the preparative synthesis of uridine 5´-triphosphate-[5-^{3}H] in high yield may be recommended mainly for the substantial shortening of the incubation period avoiding simultaneously the possible degradation of unstable compounds.

REFERENCES

1. Čihák A., Seifertová M., and Veselý J. - Coll. Czech. Chem. Commun., 36: 2321 (1971).
2. Filip J., Hybš K., Nejedlý Z., Greger J., Veselý J., and Čihák A. - J. Labelled Compounds 8: 25 (1972).
3. Čihák A., Veselý J., and Harrap K.R. - Biochem. Pharmacol., in press.
4. Čihák A., and Veselý J. - J. Biol. Chem. 248: 1307 (1972).
5. Čihák A., in preparation.
6. Nejedlý Z., Hrušková S., Hybš K., Filip J., Veselý J., and Čihák A. - J. Labelled Compounds 7: 69 (1971).

7. Škoda J., Hess V.F., and Šorm F. - Coll. Czech. Chem. Commun. 22: 1130 (1957).

8. Canellakis E.S., Gottesman M.E., and Kammen H.O. - Biochim. Biophys. Acta, 39: 82 (1960).