Journal of Labelled Compounds — October-December — Vol. V, No. 4

New Possibilities of Enzymic Synthesis of Radioactive Nucleotides.

I. Synthesis of Radioactive Nucleoside-5′-Triphosphates by an Enzyme of *Escherichia coli B*

Zdeněk NEJEDLÝ, Jindřich EKL, Karel HYBŠ and Jiří FILIP

Institute for Research, Production and Uses of Radioisotopes, Přístavní 24, Prague 7, Czechoslovakia.

Received May 25, 1969.

SUMMARY

Possibilities of enzymic synthesis of nucleoside-5′-triphosphates-$^{14}C(U)$ of adenine, guanine, cytosine and uracil of high specific activity and radiochemical purity by means of a purified enzyme isolated from Escherichia coli *B were studied. The effect of enzyme concentration in the reaction mixture in dependence on the time of the enzyme reaction is decisive for an optimal production of radioactive nucleoside-5′-triphosphates. A simple and rapid method of preparation of radioactive nucleoside-5′-triphosphates, including preparative paper chromatography as the single isolation step, is described and information on the radiolysis of the isolated nucleotides is presented.*

INTRODUCTION.

Nucleoside 5′-triphosphates play an important role in the mechanism of some biological processes in the living cell. In particular, the finding that they are direct precursors for the synthesis of RNA and DNA results in further interest in these compounds. For an ever increasing number of experiments of biochemical and biological character one needs nucleoside 5′-triphosphates labelled with ^{14}C, of high specific activity and radiochemical purity.

At the present time, we have information on the problems involved as might facilitate the elaboration of an effective method for the preparation of radioactive nucleoside 5′-triphosphates. The original, generally recognized,

procedures of chemical synthesis of nucleoside-5′-triphosphates [1-4], although yielding fine results under optimal conditions (up to 80 % yield) [3-4], are not quite suitable for the present purpose. First of all because the experiments are carried out in amounts of 1 mmole of the starting 5′-mononucleotide, this resulting in an adaptation of the isolation and separation techniques. It would be necessary to interfere seriously with these procedures if it were at all possible to prevent substantial losses of radioactive material, the amounts of the radioactive substance taken into the preparation being of the order of several micromoles.

The enzyme procedures appear to be more suitable for the synthesis of radioactive nucleoside-5′-triphosphates, primarily for the following reasons (*a*) a several-step organic synthesis can be replaced with a single reaction of the radioactive substrate with a "donor" of the phosphate group, using catalysis by a suitable enzyme preparation, (*b*) the enzyme preparation can be obtained at sufficient enzyme activity and in a sufficiently pure state so that the reactions then take place in favour of formation of nucleoside-5′-triphosphates, (*c*) methods of isolation of products are much simpler than those of organic synthesis.

One of the possibilities of enzyme synthesis of nucleoside-5′-triphosphates, mentioned by Canellakis [5, 6], consists in the ability of the dialyzed supernatant of a homogenate of regenerating rat liver to catalyze phosphorylation of some bases, nucleosides and nucleoside-5′-monophosphates of nucleic acids. Recently a procedure for an enzyme synthesis of radioactive 5′-nucleoside di- and triphosphates-^{14}C was described applying for the phosphorylation of the mononucleotides-^{14}C an enzyme isolated from normal rat liver [7].

An enzyme system generally used for syntheses of this type is that from *Escherichia coli* B. Several methods of purification of this enzyme have been described [8-12]. Phosphorylation of nucleoside-5′-monophosphates of adenine, guanine, cytosine, uracil and thymine in the system of purified *E. coli* enzyme was used by Canellakis and co-workers [12] to prepare the corresponding nucleoside-5′-triphosphates; conversion of ribonucleoside 5′-monophosphates proceeded up to 51-95 %.

In all the above cases the isolation technique used was ion-exchange chromatography but the preparation of chromatographically pure compounds and the kinetic aspects are not solved in this connection.

In the present communication we shall report on some data obtained in experiments with the phosphorylation of ribonucleoside-5′-monophosphates-^{14}C(U) in a system containing the purified *E. coli* B enzyme. Our attention was focussed on the investigation of reaction conditions which affect significantly the course and the result of phosphorylation. A simple and rapid method of preparation of ribonucleoside-5′-triphosphates labelled non-specifically with ^{14}C of high specific activity and radiochemical purity has been developed.

MATERIALS.

Inactive chemicals.

In all the experiments described here we used the following nonlabelled compounds : ribonucleosides of guanine, adenine, cytosine and uracil and the sodium or barium salts of ribonucleoside-5'-monophosphates and ribonucleoside-5'-diphosphates of these bases, obtained from Calbiochem (USA). The sodium salts of cytidine 5'-triphosphate and adenosine 5'-triphosphate were commercial products of Reanal (Hungary) whereas uridine-5'-triphosphate and guanosine-5'-triphosphate were obtained again from Calbiochem, just as creatine phosphate (disodium salt with $4H_2O$) and creatine phosphokinase from rabbit muscle. All the other nonlabelled compounds were obtained from Lachema (Czechoslovakia), mostly in a reagent-grade quality.

Labelled compounds.

As radioactive substrates we used ribonucleoside-5'-monophosphates-$^{14}C(U)$ of adenine, guanine, cytosine and uracil of specific activity at an average of 150-300 mCi/mmole. All the labelled compounds used were prepared in this laboratory according to previously developed procedures [13, 14].

Enzyme preparation of Escherichia coli *B.*

(*a*) The first of the enzyme preparations used (subsequently designated as E*1*) was prepared by the method of Canellakis [12]. The purified preparation was analyzed for protein (photometrically, by the biuret reaction at 555 nm) which was found to be present at a concentration of 28 mg/ml solution.

(*b*) The second enzyme preparation used (subsequently designated as E*2*) was prepared analogously with a modification in the cultivation phase of the bacterial culture. Five liters of a nutrient solution containing only glucose were inoculated with 150 ml culture of *E. coli* B 24 hours old. Under intense aeration, the culture was cultivated for 12-24 h at 37° C. Growth was interrupted by cooling. The cells were centrifuged, washed with 0.9 % solution of sodium chloride cooled to 3° C and frozen. This yielded 13.0 g frozen cells which were then suspended in 45 ml cold 0.01M potassium phosphate buffer of pH 7.4 and gradually desintegrated by sonication in a MSE apparatus run at maximum output (60 W, $2 . 10^4$ cycles/sec), for 5 min at 1-3° C. Centrifugation of the suspension at 10,000 r.p.m. at 1-3° C for 30 min removed cell debris and produced an opalescent supernatant (49 ml). The enzyme present in this supernatant was further purified according to Canellakis [12]. A total of 22.5 ml dialyzate containing 26.0 mg protein/ml was obtained.

Both enzyme preparations were frozen and kept at −15° C.

METHODS.

Paper Chromatography.

Selected chromatographic systems were tested for their suitability in separating products of the enzyme reactions studied. We used then *n*-butanol : acetone : acetic acid : 5 % ammonium hydroxide : water (9 : 3 : 2 : 2 : 4) which is recommended by Randerath and Struck [15] both for paper chromatography and for thin-layer chromatography, and further isobutyric acid : water : concentrated ammonia (66 : 33 : 1.5) [16]. The chromatograms were run on Whatman No. 3 at room temperature for 20 h. in the descending direction. The R_f values on Whatman No. 3 are shown in Table 1.

Paper electrophoresis.

As a simple and rapid method for checking the course of the enzyme reactions and for verifying the enzyme activity we used paper electrophoresis. This was done in 0.05 M citrate buffer of pH 3.8 in an arrangement according to Markham and Smith [17]. The deproteinized samples of the reaction mixture are placed on Whatman No. 3 paper 57 cm long and 20 cm wide,

TABLE 1. Paper chromatography of ribonucleosides and ribonucleoside 5'-mono-, di- and triphosphates of adenine, guanine, uracil and cytosine. R_F values are shown.

Compound	Solvent System	
	A	B
AR	0.68	0.62
AMP	0.61	0.27
ADP	0.50	0.16
ATP	0.38	0.11
CR	0.66	0.46
CMP	0.48	0.24
CDP	0.34	0.15
CTP	0.25	0.09
GR	0.58	0.44
GMP	0.34	0.17
GDP	0.24	0.08
GTP	0.17	0.05
UR	0.64	0.49
UMP	0.34	0.25
UDP	0.22	0.17
UTP	0.15	0.10

A = isobutyric acid-water-concentrated ammonia (66 : 33 : 1.5).
B = *n*-butanol-acetone-acetic acid-5 % ammonia-water (9 : 3 : 2 : 2 : 4).

15 cm from the cathode. The paper is then impregnated with the buffer and electrophoresis is carried out in a carbon tetrachloride bath with an initial voltage of 500 V, current density 18 mA, for 3.5 h. The distances travelled by the individual nucleotides and nucleosides are shown in Table 2.

Detection methods.

The radioactive compounds were detected by autoradiography using Agfa X-ray films. The radiochromatograms and radioelectrophoretograms were also evaluated on a Frieseke-Hoepfner counter. Inactive standards of the nucleic acid components were detected in the UV light of Chromatolite.

Determination of radioactivity of ^{14}C and UV spectrophotometry.

The radioactivity of ribonucleoside-5′-triphosphates-^{14}C(U) was determined by scintillation after conversion to ethanolic solutions. To increase the solubility of the sample (ethanol-water) we used a liquid scintillator containing dioxane SLD 31 (Tesla Pardubice). The detection efficiency was tested by the method of internal standard (EK-2^{14}C toluene, produced at this Institute). The error of determination of specific activity of the ethanolic solution is less than 10 %.

The content of ribonucleoside-5′-triphosphates in the final solutions (50 % ethanolic solution of 0.75 M ammonia) was determined spectrophotometrically in UV light. Concentration of the triphosphates in these solutions if read from calibration curves obtained by measuring the extinction of the individual triphosphates at their absorption maximum.

EXPERIMENTAL.

Course of phosphorylation of nucleoside-5′-monophosphates-^{14}C catalyzed by a purified E. coli *B enzyme system (E1).*

The catalytic efficiency of the *E. coli* B enzyme (E*1*) for the phosphorylation of radioactive-5′-mononucleotides-^{14}C was checked by incubation of a mixture

TABLE 2. Paper electrophoresis of nucleotides. The mobilities of nucleotides are expressed in M_G values in relation to the mobility of UTP = 1.0.

Compound	M_G	Compound	M_G
5′-AMP	0.295	5′-GMP	0.498
5′-ADP	0.603	5′-GDP	0.773
5′-ATP	0.798	5′-GTP	0.908
5′-CMP	0.278	5′-UMP	0.518
5′-CDP	0.655	5′-UDP	0.873
5′-CTP	0.828	5′-UTP	1,00

Conditions of paper electrophoresis are described in the text.

of AMP-^{14}C(U) or GMP-^{14}C(U) (1 μmole, 40 μCi), ATP (0.46 μmole), $MgCl_2 \cdot 6H_2O$ (5.1 μmole), creatine phosphate (5.9 μmole) and creatine kinase (0.03 mg) in a total volume of 0.4 ml of 0.02 M glycine buffer of pH 7.5 with 0.03 ml freshly obtained enzyme preparation, at 37° C under aseptic conditions. Electrophoresis of the samples of the reaction mixture after 10 and 20 min determined the distribution of radioactivity of ^{14}C in the individual reaction products (see Table 3*a*, *b*).

Similarly, using the same enzyme preparation but after 6 months of storage at −10° C, a series of experiments was conducted to examine the possibility of enzyme phosphorylation of 5′-mononucleotides-^{14}C (see Table 4*a*-*e*). In this case we incubated at 37° C under aseptic conditions a mixture of AMP-^{14}C (or GMP-^{14}C(U), CMP-^{14}C(U), UMP-^{14}C(U)) (1.3 μmole, 40 μCi), ATP (0.6 μmole), $MgCl_2 \cdot 6H_2O$ (6.7 μmole), creatine phosphate (7.7 μmole) and creatine kinase (0.04 mg) in a volume of 0.5 ml 0.02 M glycine buffer of pH 7.5 using either 0.3 ml (Table 4*a*-*d*) or 0.1 ml (Table 4*e*) of the enzyme preparation. Under otherwise identical molar ratios of the individual components of the reaction mixture the phosphorylation was thus carried out with a six or two times higher amount of the enzyme. Samples of the reaction mixture taken during the 60 or 180 min reaction periods were analyzed by electrophoresis.

On the basis of the results of the experiments reported here one can draw several conclusions. It appears that the application of even very small amounts of freshly prepared enzyme, such as 0.04 ml (1 μmole of radioactive substrate) results under suitable conditions in a high conversion of the substrate to the nucleoside-5′-triphosphate, the formation of the mono- and diphosphates, just as the dephosphorylation of the substrate to the nucleoside, being negligible. It may be further seen that the enzyme activity of the preparation

TABLE 3. Course of phosphorylation of 5′-AMP-^{14}C(U) (*a*) and 5′-GMP-^{14}C(U) (*b*) in a system containing the purified *E. coli* B enzyme (E*1*)[a]. Distribution of radioactivity of the electrophoretogram is expressed as % of total radioactivity of the reaction mixture.

(*a*) Compound	Reaction period (min)		(*b*) Compound	Reaction period (min)	
	10	20		10	20
AR-^{14}C	3.2	4.1	GR-^{14}C	4.8	5.3
5′-AMP-^{14}C	1.8	4.0	5′-GMP-^{14}C	5.2	5.8
5′-ADP-^{14}C	3.6	3.4	5′-GDP-^{14}C	6.8	9.9
5′-ATP-^{14}C	91.4	88.5	5′-GTP-^{14}C	83.2	79.0

[a] The reaction conditions are described in the text.

is sufficient so that even after 6 months of storage the phosphorylation proceeded in favour of the purine and pyrimidine nucleoside-5′-triphosphates. Nevertheless, satisfactory results are obtained only after application of a six-fold amount of the enzyme preparation; in the presence of only a two-fold

TABLE 4. Course of phosphorylation of 5′-AMP-^{14}C(U) (*a*), 5′-GMP-^{14}C(U) (*b*), 5′-CMP-^{14}C(U) (*c*), and 5′-UMP-^{14}C(U) (*d, e*) in a system containing the purified enzyme from *E. coli* B (E*I*) [a].
Distribution of radioactivity on the electrophoretograms is expressed in % of total radioactivity of the reaction mixture.

Compound	Reaction period (min)					
	10	20	40	60	120	180
(*a*)						
AR-^{14}C	4.8	5.8	7.0	8.2	—	—
5′-AMP-^{14}C	7.8	11.6	21.9	38.8	—	—
5′-ADP-^{14}C	23.0	25.9	30.3	29.4	—	—
5′-ATP-^{14}C	64.6	56.8	40.4	23.7	—	—
(*b*)						
GR-^{14}C	16.7	23.1	32.0	36.2	—	—
5′-GMP-^{14}C	14.5	11.3	6.6	6.5	—	—
5′-GDP-^{14}C	15.1	15.8	16.7	19.6	—	—
5′-GTP-^{14}C	53.7	49.7	44.8	37.8	—	—
(*c*)						
CR-^{14}C	12.2	15.0	21.6	27.8	—	—
5′-CMP-^{14}C	13.1	10.2	4.0	2.9	—	—
5′-CDP-^{14}C	24.6	30.0	38.9	43.1	—	—
5′-CTP-^{14}C	50.2	44.8	35.6	26.2	—	—
(*d*)						
UR-^{14}C	3.2	4.9	5.3	9.2	—	—
5′-UMP-^{14}C	5.2	7.8	12.2	18.1	—	—
5′-UDP-^{14}C	12.3	24.9	33.5	40.2	—	—
5′-UTP-^{14}C	79.3	62.5	48.8	32.6	—	—
(*e*)						
UR-^{14}C	3.6	5.3	5.4	9.2	11.7	19.1
5′-UMP-^{14}C	58.2	43.7	24.3	14.7	11.7	31.6
5′-UDP-^{14}C	8.8	14.7	26.9	33.3	41.2	37.1
5′-UTP-^{14}C	29.3	36.5	43.4	43.0	35.5	12.2

[a] Reaction conditions are described in the text.

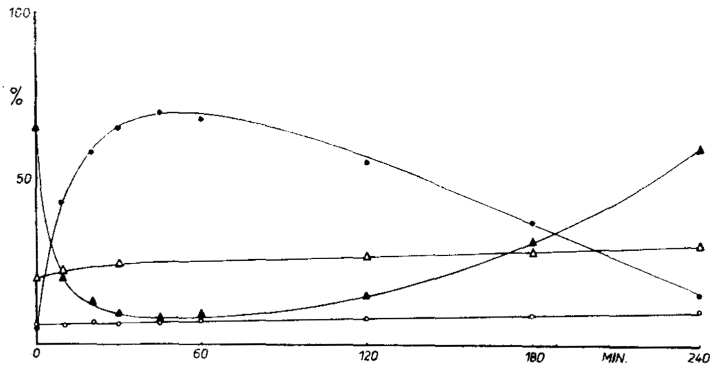

FIG. 1. Course of phosphorylation of 5'-AMP-^{14}C(U) in a system containing the purified *E. coli* B enzyme (E*2*).

The mixture of 5'-AMP-^{14}C(U) (1.3 μmole; 20 μCi), ATP (11.3 μmole), $MgCl_2 \cdot 6H_2O$ (13.3 μmole), creatine phosphate (15,4 μmole) and creatine kinase (0.08 mg) in a total volume of 0.6 ml of 0.02 M glycine buffer of pH 7.5 was incubated with 0.2 ml of enzyme E*2* (stored 4 months) at 37° C under aseptic conditions.

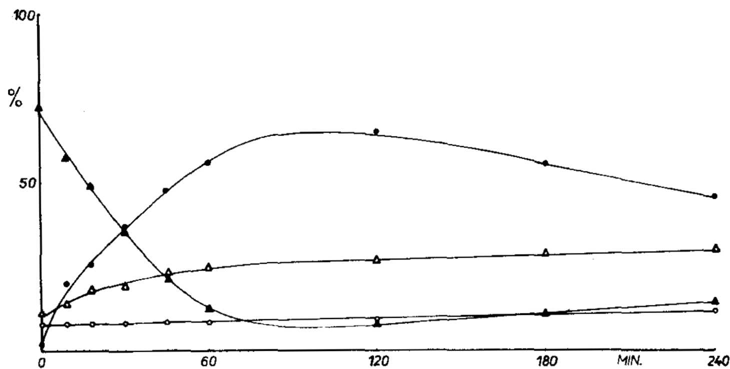

FIG. 2. Course of phosphorylation of 5'-AMP-^{14}C(U) in a system containing the purified *E. coli* B enzyme (E*2*).

The mixture of 5'-AMP-^{14}C(U) (1.3 μmole; 20 μCi), ATP (11.3 μmole), $MgCl_2 \cdot 6H_2O$ (13.3 μmole), creatine phosphate (15.4 μmole) and creatine kinase (0.08 mg) in a total volume of 0.6 ml of 0.02M glycine buffer of pH 7.5 was incubated with 0.6 ml of enzyme E*2* (stored 4 months) at 37° C under aseptic conditions.

amount of enzyme the synthesis of UTP-^{14}C is only about one half that with the higher amount of enzyme. The purine and pyrimidine nucleoside-5′-triphosphates are dephosphorylated in time. Whereas in the case of UTP and ATP the first phases of the reaction result in dephosphorylation to 5′-mononucleotides, with GTP and CTP the dephosphorylation proceeds appreciably all the way to nucleosides.

Effects of concentration of the E. coli *B enzyme (E2) on the course of phosphorylation of nucleoside 5′-monophosphates.*

In addition to a suitable concentration and mutual ratios of ATP, the ATP-regenerating system and radioactive substrate in the reaction mixture it is the enzyme concentration which determines the course of the enzyme reaction and the degree of substrate conversion to the nucleoside-5′-triphosphate. Figures 1-3 show the effect of different concentrations of the catalyzing enzyme on the course of phosphorylation and mutual conversion of 5′-nucleotides of adenosine-^{14}C. In this experiment the mixture of 5′-AMP-^{14}C(U) (1.29 μmole, 20 μCi), ATP (11.28 μmole), $MgCl_2 \cdot 6H_2O$ (13.3 μmole), creatine phosphate (15.4 μmole) and creatine kinase (0.08 mg) in 0.6 ml 0.02 M glycine buffer of pH 7.5 was incubated at 37° C under aseptic conditions with (*a*) 0.2 ml, (*b*) 0.6 ml and (*c*) 1.0 ml enzyme preparation E*2*, stored for 4 months. Samples of the reaction mixtures removed in the course of the 240 min reaction periods were analyzed electrophoretically. Graphical records (see Figs. 1-3) show a clear difference of the course of the enzymic phosphorylation, with regard to the degree of conversion AMP→ATP, a temporal shift of the conversion maximum and the degree and course of the back dephosphorylation of ATP to mono- and diphosphate and adenosine. From the preparatory point of view, optimal yields are obtained by application of 0.6 ml enzyme preparation E*2* under the above conditions. It was just under such conditions that the phosphorylation of UMP-^{14}C(U) in this enzyme system was examined (Fig. 4). The distribution of radioactivity of ^{14}C at the moment of optimum conversion of AMP→ATP (UMP→UTP) in the individual reaction products is thus in favour of formation of nucleoside 5′-di- and triphosphates-^{14}C(U).

Enzyme synthesis of nucleoside-5′-triphosphates-$^{14}C(U)$ of adenine, guanine, uracil and cytosine on a semipreparative scale.

A mixture of 5′-AMP-^{14}C(U) (5′-GMP-^{14}C(U), (5′-UMP-^{14}C(U), 5′-CMP-^{14}C(U) (40 μCi, 0.25 μmole), ATP (0.44 μmole), $MgCl_2 \cdot 6H_2O$ (4.8 μmole), creatine phosphate (5.3 μmole) and creatine kinase (0.056 mg) in a volume of 0.4 ml 0.02 M glycine buffer was incubated with 0.07 ml purified enzyme preparation (E1) stored for 4 weeks, for a period of 40 min at 37° C under aseptic conditions.

After termination of the reaction the solutions were overlayered with an equal volume of 96 % ethanol and cooled to 0° C for 2 h. The precipitate of

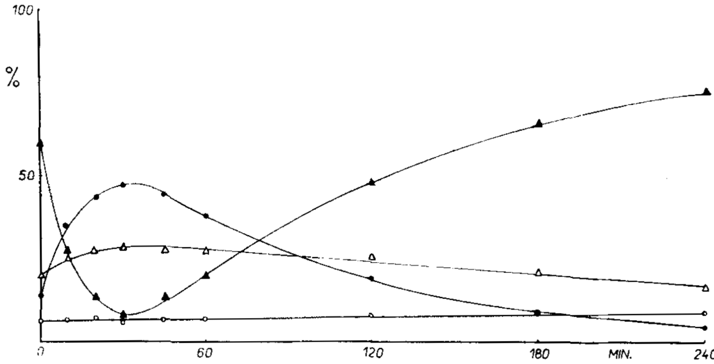

FIG. 3. Course of phosphorylation of 5′-AMP-^{14}C(U) in a system containing the purified *E. coli* B enzyme (E2).

The mixture of 5′-AMP-^{14}C(U) (1.3 μmole; 20 μCi), ATP (11.3 μmole), $MgCl_2 \cdot 6H_2O$ (13.3. μmole), creatine phosphate (15.4 μmole) and creatine kinase (0.08 mg) in a total volume of 0.6 ml of 0.02 M glycine buffer of pH 7.5 was incubated with 1.0 ml of enzyme E2 (stored 4 months) at 37° C under aseptic conditions.

▲——▲ 5′-AMP-^{14}C(U)
△——△ 5′-ADP-^{14}C(U)
●——● 5′-ATP-^{14}C(U)
○——○ AR-^{14}C(U)

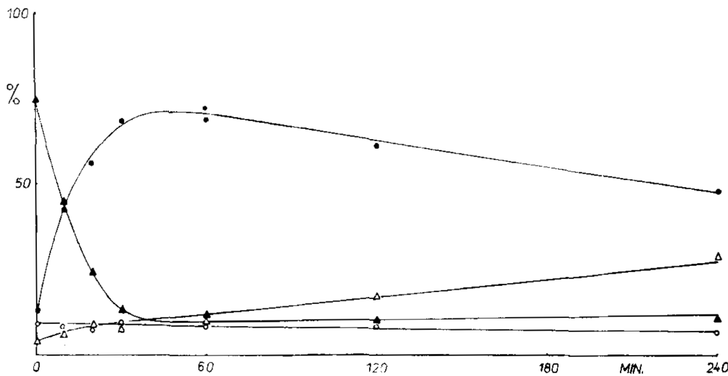

FIG. 4. Course of phosphorylation of 5′-UMP-^{14}C(U) in a system containing the purified *E. coli* B enzyme (E2).

The mixture of 5′-UMP-^{14}C(U) (1.3 μmole; 20 μCi), ATP (11.3 μmole), $MgCl_2 \cdot 6H_2O$ (13.3 μmole), creatine phosphate (15.4 μmole) and creatine kinase (0.08 mg) in a total volume of 0.6 ml of 0.02M glycine buffer of pH was 7.5 incubated with 0.6 ml enzyme E2 (stored 4 months) at 37° C under aseptic conditions

▲——▲ 5′-UMP-^{14}C(U)
△——△ 5′-UDP-^{14}C(U)
●——● 5′-UTP-^{14}C(U)
○——○ UR-^{14}C(U)

protein was centrifuged (15 min, 6,000 r.p.m.) and the supernatant fraction was chromatographed on Whatman No. 3 together with standards of the corresponding nucleosides and nucleotides using a system of isobutyric acid : water : concentrated ammonia (66 : 33 : 1.5). Chromatographic papers were dried for 34 h in a current of cold air in an ammonia atmosphere and rechromatographed in *n*-butanol saturated with water. In this way all of the isobutyric acid was removed from the paper. Components of nucleic acids with the exception of ribonucleosides do not move in this solvent system. The chromatographic papers were again dried in a current of cold air and radioautography was carried out. Strips corresponding to ribonucleoside-5'-triphosphate were cut out and the radioactive compounds eluted with a mixture of 96 % ethanol : 1.5 N ammonia (1 : 1) overnight at room temperature. The total radioactivity of ^{14}C and the specific activity of the eluted nucleoside-5'-triphosphates-^{14}C(U) was determined. The yields referred to the starting radioactivity of the substrate are all higher than 70 % : ATP-^{14}C (85.2 %) GTP-^{14}C (81.1 %); CTP-^{14}C (71.3 %), UTP-^{14}C (76.8 %). The specific activity of the triphosphates with the exception of ATP-^{14}C (60 mCi/mmole) varies about 200 mCi/mmole; GTP-^{14}C (188 mCi/mmole), CTP-^{14}C (197 mCi/mmole), UTP-^{14}C (218 mCi/mmole).

TABLE 5. Stability of ribonucleoside-5'-triphosphates-^{14}C(U) in ethanol-1.5N ammonia (1 : 1) at —10° C.

Compound	Period of storage after isolation Days	Radio-chemical purity %	Content of radiochemical impurities [a] %		
			Total	5'-Mono-phosphates	5'-Di-phosphates
5'-ATP-^{14}C(U)	7	98.2	1.8	—	1,8
5'-GTP-^{14}C(U)	7	98.2	1.8	—	1.8
5'-CTP-^{14}C(U)	7	97.9	2.1	—	2.1
5'-UTP-^{14}C(U)	7	94.3	5.7	—	5.7
5'-ATP-^{14}C(U)	14	95.8	4.2	0.3	3.9
5'-GTP-^{14}C(U)	14	95.5	4.5	0.7	3.8
5'-CTP-^{14}C(U) ...	14	95.4	4.6	—	4,6
5'-UTP-^{14}C(U)	14	90.0	10.0	—	10.0

[a] Analyzed in isobutyric acid-water-ammonia (66 : 33 : 1.5).

Radiochemical stability of nucleoside-5′-triphosphates-$^{14}C(U)$.

The ribonucleoside-5′-triphosphates of adenine, guanine, uracil and cytosine are compounds highly unstable chemically. If all the carbons in the molecule of the triphosphate are labelled with ^{14}C one must moreover assume the role of the radiolytic effect. This effect will be emphasized in the given case by the fact that the nucleoside-5′-triphosphates-$^{14}C(U)$ prepared here are compounds of high specific radioactivity. For these reasons and because of the applicability of such preparations in biochemical and biological studies we tested in an orientation manner their stability during the assumed period of their storage before application. The preparations were maintained for two weeks in a solution of ethanol-1.5 N ammonia (1 : 1) at -10° C. After 1 and 2 weeks they were analyzed by paper chromatography (Table 5). As may be seen from the results, under the conditions of storage the stability is such that even after two weeks it is satisfactory for application. With the exception of UTP-^{14}C, radiochemical purity higher than 95 % was estimated with all the triphosphates. Radiolysis takes place mostly to 5′-diphosphates and only in the case of the purine nucleotides we detected after two weeks a small amount of 5′-monophosphates.

CONCLUSIONS.

The enzyme phosphorylation of ribonucleoside-5′-monophosphates-$^{14}C(U)$ catalyzed by the presence of a purified enzyme system from *Escherichia coli* B represents a simple and rapid method of production of radioactive ribonucleoside-5′-triphosphates-$^{14}C(U)$ with high yields.

The relatively low substrate specificity of the enzyme makes it possible to phosphorylate both purine and pyrimidine 5′-mononucleotides. Under the constant conditions used (molar ratio of radioactive substrate, ATP and the ATP-regenerating system) the enzyme concentration and the duration of the incubation represent the decisive factors from the point of view of optimal production of radioactive nucleoside-5′-triphosphates. Due to the presence of phosphatases activity in the enzyme preparation the enzyme reactions are accompanied by a dephosphorylation of the nucleoside-5′-triphosphates down to 5′-mononucleotides, the dephosphorylation to the ribosides being minimal. The dephosphorylation is quite pronounced in the presence of excess enzyme preparation.

The stability of the enzyme preparation is satisfactory. E.g., even after six months of storage the preparation still achieved fine phosphorylation. It must be noted, however, that on applying older enzyme preparations the dephosphorylation proceeds clearly all the way to the ribosides, particularly in the case of guanine and cytosine nucleotides. Hypothetically, this can be explained probably by a different course of deactivation of various enzyme activities in the course of storage of the enzyme or by a lower stability of the synthetase activity as compared with the hydrolase one.

From the preparative point of view, it is advantageous that nucleoside-5′-monophosphates are converted to the nucleoside-5′-triphosphates in a high degree, all the other products of reaction being also biologically important and commercially useful. The favourable composition of the final reaction mixtures makes it possible to apply preparative paper chromatography as the single isolation procedure. In this way, the whole preparative procedure is highly simplified and shortened and the undesirable degradation of the nucleoside-5′-triphosphates during their preparation is prevented.

The information obtained on the radiolysis of the nucleoside-5′-triphosphates indicates that their immediate application to chemical and biological purposes is desirable. Nevertheless, other ways of storage of these compounds as are now investigated in this laboratory, indicate much better possibilities even in this respect.

REFERENCES

1. Khorana, H. G. — *J. Am. Chem. Soc.*, **76** : 3517 (1954).
2. Moffatt, J. G. and Khorana, H. G. — *J. Am. Chem. Soc.*, **83** : 649 (1961).
3. Moffatt, J. G. — *Can. J. Chem.*, **42** : 599 (1964).
4. Michelson, A. M. — *Biochem. Biophys. Acta,* **91** : 1 (1964).
5. Canellakis, E. S. — *J. Biol. Chem.*, **227** : 329 (1957).
6. Canellakis, E. S. and Mantsavinos, R. — *Biochim. Biophys. Acta*, **27** : 643 (1958).
7. Maley, F., Maley, G. F. and McGarrahan, J. F. — *Anal. Biochem.*, **19** : 265 (1967).
8. Reichard, P., Baldesten, A. and Rutberg, L. — *J. Biol. Chem.*, **236** : 1150 (1961).
9. Bresler, A. E. — *Biochim. Biophys. Acta*, **61** : 29 (1962).
10. Bertani, L. S., Häggmark, A. and Reichard, P. — *J. Biol. Chem.*, **236** : PC 67 (1961).
11. Reichard, P. — *J. Biol. Chem.*, **237** : 3513 (1962).
12. Canellakis, E. S., Gottesman, M. E. and Kammen, H. O. — *Biochim. Biophys. Acta*, **39** : 82 (1960).
13. Nejedlý, Z., Koutecký, Z. and Grünberger, D. — *Coll. Czech. Chem. Commun.*, **30** : 3361 (1965).
14. Nejedlý, Z., Filip, J. and Grünberger, D. — Proceedings of the Second International Conference on Methods of Preparing and Storing labelled Compounds, Brussels, November 28. Dec. 3, 1966.
15. Randerath, K., Struck, H. — *J. Chromatog.*, **6** : 365 (1961).
16. Magasanik, B., Vischer, E., Doniger, R., Elson, D. and Chargaff, E. — *J. Biol. Chem.*, **186** : 37 (1950).
17. Markham, R. and Smith, J. — *Biochem. J.*, **52** : 522 (1952).