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New Possibilities of Enzymic Synthesis of Radioactive Nucleotides

III. Preparative Synthesis of Radioactive 5′-Nucleotides by an Unpurified Cell-Free Extract of Mouse Leukemic Cells

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SUMMARY

AKR leukemic mouse cells possess a considerable uridine kinase activity. The ability of unpurified cell-free extracts prepared from these cells to phosphorylate nucleosides-^{14}C was investigated. The substrate specificity of the enzyme preparation and the possibility to regulate the course of the enzymatic reactions were examined. The method is convenient for the preparative synthesis of uracil, cytosine and hypoxanthine 5′-nucleotides-^{14}C.

I. — INTRODUCTION

The study of simple and effective procedures for the preparation of radioactive nucleotides represents an important field of the applied biochemistry. The use of enzyme preparations for the synthesis of these substances is of particular value. In the present communication we wish to report on some of the results obtained in these laboratories with cell-free preparation from mouse leukemic cells.

In our previous work we have described a convenient and efficient method which consists in using the purified enzymatic preparation from *E. coli B* [1] for the synthesis of nucleoside-5′-triphosphates-^{14}C with high specific activity. In another instance phosphoribosylation of orotic acid-^{14}C and uracil-^{14}C

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by the cell-free extract from *Brevibacterium ammoniagenes* to orotidine-5'-phosphate-^{14}C and uridine-5'-monophosphate-^{14}C[2,3] in a very considerable yield was obtained.

It is known that mouse leukemic cells possess an enhanced activity of different enzymes [4], and could thus seem to be particularly suitable for the preparation of enzymatic samples that could be used for the synthesis of various radioactive nucleotides at a larger scale. The cell-free extracts from mouse leukemic livers contain in the terminal stage of the disease (when the number of leukemic lymphocytes increases [5] at the expense of specific liver parenchymal cells) a sufficient amount of kinases [6, 7] which are responsible for the phosphorylation of nucleosides since leukemic lymphocytes are able to utilize efficiently the preformed precursors of nucleic acids along the salvage pathway. During the study of metabolism of leukemic cells enhanced uridine kinase activity has been observed [7-9]. In the present report this phenomenon has been studied more in detail, especially in view of its practical application which would lead to the synthesis of radioactive nucleotides.

II. — Materials

A. — *Chemicals.*

Unlabelled components of nucleic acids were products of Calbiochem (U.S.A.) which delivered also the rabbit muscle creatine kinase (lyophilized powder) and creatine phosphate. Other chemicals (non-radioactive) were purchased from Lachema (Czechoslovakia) in analytically pure form. Radionuclides labelled with ^{14}C are commercial samples of the Institute for Research, Production and Uses of Radioisotopes in Prague. The specific activity of nucleosides labelled specifically with ^{14}C is 44 mCi/mmole, while of those labelled nonspecifically it is 200 mCi/mmole. Radiochemical purity of substances exceeds 98 %.

B. — *Enzymic preparation.*

As a source of enzyme activity the supernatant fraction from the sonicate of mouse leukemic livers was used. Livers were homogenized in 3 volumes of ice-cold 0.01M Tris-HCl buffer (pH 8.1) with 1.15 % KCl in a glass homogeniser with a motor-driven (1,200 r.p.m.) plexiglass pestle. Homogenate was subjected to sonication (MSE ultrasonic disintegrator) for 10-60 seconds, at 10-20 $\times$ 10^3 cycles/sec. The sonicate was centrifuged (Spinco ultracentrifuge) and the supernatant fraction was used directly as a source of corresponding enzymes. No loss of the activity was observed for 1 month upon storage at —20° C. However, in different sets of experiments fresh enzyme preparations were used. Protein content (generally 20-30 mg/ml) was determined according to Lowry *et al.* [10].

III. — METHODS

A. — *Reaction conditions.*

As radioactive substrates uridine-, cytidine-, guanosine- and adenosine-^{14}C were employed. As the donor of phosphate groups adenosine-5′-triphosphate in the presence of Mg^{2+}-ions was used. Reactions were carried out in the presence of 0.02M Tris-HCl buffer (pH 8.1). Incubation period was terminated by layering the reaction mixture over with 2 volumes of 50 % ethanol (+2° C) for 2 h in the cold. Precipitated proteins were discarded and the clear solution was directly analysed.

B. — *Separation of the reaction products.*

The reaction products were separated by means of descending paper chromatography in the solvent systems composed of isobutyric acid : water : ammonium hydroxide (66 : 33 : 1.5), and/or water-saturated butanol using Whatman paper No. 3 at room temperature. Identity of the products was determined by simultaneous chromatography of standards. Besides paper chromatography, paper electrophoresis according to Markham and Smith [11] for the first orientation was used in some cases.

C. — *Detection.*

Radioactive substances were detected by autoradiography with ORWO X-Ray films, while the standards were identified in ultraviolet light (Chromatolite). Quantitative evaluation of chromatograms was carried out with Frieseke-Hoepfner scanner and planimetry. The total radioactivity of 5′-nucleotides-^{14}C was assayed with liquid scintillation counting.

The concentration of radioactive 5′-nucleotides-^{14}C in reaction mixtures (50 % ethanol solution of 0.75M NH_4OH) was determined by spectrophotometry in the ultraviolet region on the basis of calibration curves at λ_{max} for individual 5′-nucleotides.

IV. — RESULTS

A. — *The determination of conditions for optimal uridine-U-^{14}C phosphorylation.*

Uridine-^{14}C was chosen as a radioactive substrate for the initial studies of phosphorylation of nucleosides in the presence of cell-free extracts from mouse leukemic cells. Its phosphorylation was carried out under following "standard" conditions : Uridine-^{14}C(U), 0.1 μmole; ATP, 2.5 μmole; and Mg^{2+}-ions, 2.5 μmole were dissolved in 0.4 ml of 0.02M Tris-HCl buffer (pH 8.1) and incubated at 37° C with 0.1 ml of the enzyme which was obtained following 1 min sonication and centrifugation at 50,000xg (1 hr).

Figure 1 indicates the time course of the reaction; its main product is uridine-5′-monophosphate-^{14}C. The maximum of its formation takes place after 120 min (68 % of the total radioactivity). Further product is uridine-5′-triphosphate-^{14}C with maximal formation after 200 min (19 % of the total radioactivity). Besides these substances a small number of other unidentified compounds is formed.

Next experiment was carried out under identical conditions in the presence of ATP-regenerating system consisting of creatine phosphate (2.5 mg) and creatine phosphokinase (0.08 mg). Its effect on keeping ATP concentration at a relatively constant level (throughout the duration of incubation) was examined.

Under these circumstances (Fig. 2) uridine-5′-triphosphate-^{14}C is formed preferentially (maximum at 200 min, 60 % of the total radioactivity). Uridine-5′-monophosphate-^{14}C is synthesized especially during the first phase of incubation period (maximum at 50 min, 45 % of the total radioactivity). Later on the concentration of uridine-5′-monophosphate-^{14}C decreases rather abruptly. At the end of the incubation period only 5 % of the initial amount of uridine-^{14}C was present in the reaction mixture.

Next the effect of different centrifugal forces applied to the sonicate on the pattern of uridine-^{14}C phosphorylation reactions was tested. Enzyme samples were prepared by the standard procedure; however, the sonicates were centrifuged at 110,000xg or at 5,000xg for 2 h, respectively. The dif-

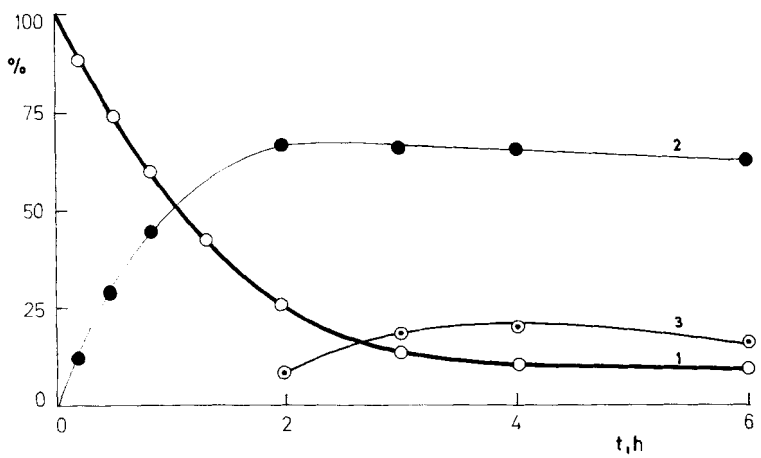

FIG. 1. Time course of uridine-^{14}C(U) phosphorylation by a cell-free extract from mice leukemic cells.

Incubation was carried out at 37° C in a total volume of 1 ml in 2 × 10^{-2}M Tris-HCl buffer (pH 8.1) with 0.1 ml of an enzyme fraction (sonication 1 min, centrifugation 1 h, 50,000xg, 2° C). 1 × 10^{-4}M uridine-^{14}C(U), 2,5 × 10^{-3}M adenosine-5′-triphosphate with equimolar Mg^{2+}-ions. 1, Uridine; 2, uridine-5′-monophosphate; and 3, uridine 5′-triphosphate in per cent of the total radioactivity (20 μCi).

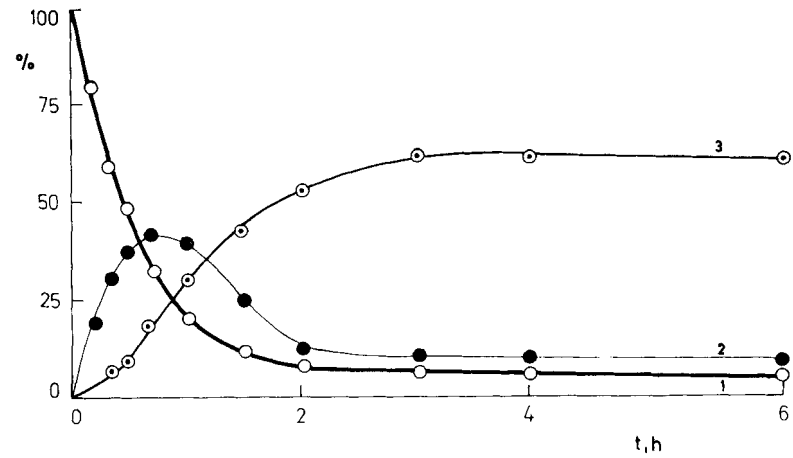

FIG. 2. Time course of uridine-^{14}C(U) phosphorylation in the presence of ATP-regenerating system.

Incubation was carried out as in Figure 1 with 0.3 ml of an enzyme fraction. 1×10^{-4}M uridine-^{14}C(U), 2.5×10^{-3}M adenosine-5′-triphosphate with equimolar Mg^{2+}-ions and 0.08 mg of creatine kinase with 2.5 mg of creatine phosphate. 1, Uridine; 2, uridine-5′-monophosphate; and 3, uridine 5′-triphosphate in per cent of the total radioactivity (20 μCi).

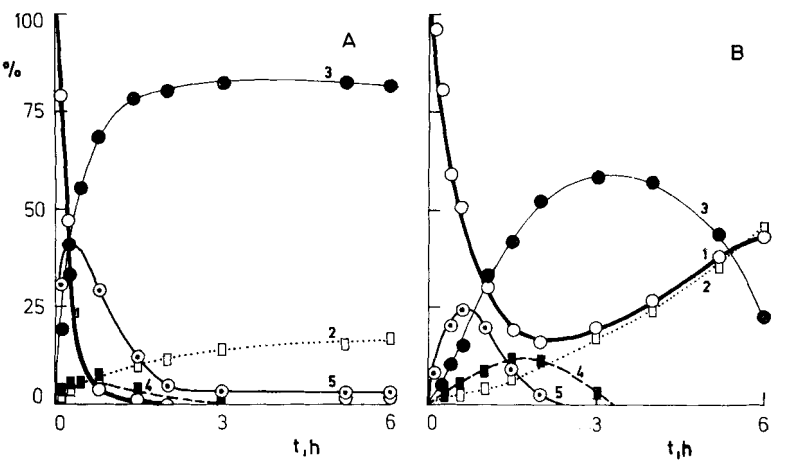

FIG. 3. Effect of centrifugation of a sonicate from mouse leukemic cells on the time course of uridine-^{14}C(U) phosphorylation.

Sonicates (1 min, 2° C, 60 sec) were centrifuged at 110,000xg, 2° C for 2 h (A) and at 5,000xg, 2° C for 2 h (B). Incubations were carried out as in Figure 1 with 0.5 ml of an enzyme fraction. 5×10^{-4}M uridine-^{14}C(U), 1.25×10^{-2}M adenosine 5′-triphosphate with equimolar Mg^{2+}-ions. 1, Uridine; 2, uracil; 3, uridine-5′-monophosphate; 4, uridine-5′-diphosphate and 5, uridine-5′-triphosphate in per cent of the total radioactivity (20 μCi).

ferent time course of the reactions is given in Figure 3A and B. The application of the soluble fraction obtained by centrifugation at 110,000xg results in an optimal yield of uridine-5′-monophosphate-^{14}C (Fig. 3A) which represents nearly 80 % of the total radioactivity. The degradation of nucleotides to uracil-^{14}C does not exceed 13 %. Uridine-5′-triphosphate-^{14}C is formed during the initial stage of incubation; it is maximal after 20 min (40 % of the total radioactivity). However, if 5,000xg supernatant fraction is used (Fig. 3B) the levels of uridine-5′-monophosphate-^{14}C and -triphosphate-^{14}C decrease, and at later phases the breakdown of nucleotides to uridine-^{14}C and uracil-^{14}C occurs.

The effect of the duration of sonication (10 and 60 sec) of the homogenate on phosphorylations was also investigated. Table 1A, B indicates that the duration of the sonication has a profound influence on the degree of the phosphorylation of 5′-nucleotides. The 10 sec-period of sonication leads to the preferential formation of uridine-5′-triphosphate-^{14}C (up to 70 %); the effect of degradative enzymes is negligible as indicated by the low content of uridine-^{14}C and uracil-^{14}C in the final reaction mixture.

TABLE 1. Effect of the duration of sonication of mouse leukemic cells on the phosphorylation of uridine-^{14}C(U) by a cell-free extract.

Incubation was carried out in 2×10^{-2}M Tris-HCl buffer, pH 8.1 at 37° C in a total volume of 1 ml with 0.1 ml of an enzyme fraction. A – sonication 10 sec; B – sonication 60 sec; centrifugation 2 h at 110,000xg. 1×10^{-4}M uridine-^{14}C(U) and 2.5×10^{-3}M adenosine-5′-triphosphate with equimolar Mg^{2+}-ions. Radioactivity of the compounds is expressed in % of the total radioactivity of the reaction mixture.

Compound	Incubation time (min)					
	A :			B :		
	15	60	120	15	60	120
UR-^{14}C	60.5	32.5	11.6	21.1	5.2	5.2
U-^{14}C	2.4	2.6	2.9	5.2	9.5	12.3
5′-UMP-^{14}C	11.4	9.1	7.7	24.9	53.1	68.4
5′-UDP-^{14}C	4.8	9.1	13.0	9.3	7.9	3.5
5′-UTP-^{14}C	20.9	46.5	64.7	37.3	25.3	8.9

B. — *Phosphorylation of other purine and pyrimidine nucleosides-^{14}C.*

Since the soluble fraction from mouse leukemic cells could be useful for the preparation of other purine and pyrimidine 5'-phosphates its substrate specificity was investigated with respect to cytidine-^{14}C and adenosine-^{14}C as well as to guanosine-^{14}C. The reaction mixtures (total volume 1 ml) contained at 37° C : nucleoside-^{14}C, 0.1 μmole; adenosine-5'-triphosphate and Mg^{2+}-ions, 2.5 μmoles; Tris-HCl buffer (pH 8.1), 4 μmoles; and 0.1 ml of enzyme (10 sec-period of sonication at maximum output of MSE ultrasonic disintegrator, 110,000xg for 2 h). The incubation lasted 3 h. The time course of incubation of cytidine-^{14}C in the presence of 2.5 and 5.0 μmoles of adenosine-5'-triphosphate is indicated in Figure 4A, B. It is obvious that the conversion of cytidine-^{14}C to cytidine-5'-triphosphate-^{14}C is relatively low. Its maximum formation (Fig. 4A) occurs at 240 min of incubation time (50 % of the total radioactivity). Cytidine-5'-monophosphate-^{14}C is formed at a minimal rate. On the other hand the concentration of cytidine-5'-diphosphate-^{14}C increases during the period of incubation steadily, and finally it represents 20 % of the total radioactivity. When the concentration of adenosine-5'-triphosphate is raised to 5 μmoles the formation of cytidine-5'-triphosphate is continuously increasing. On raising the amount of the enzyme in the reaction mixture (Table 2A, B) the formation of nucleotides diminishes substantially.

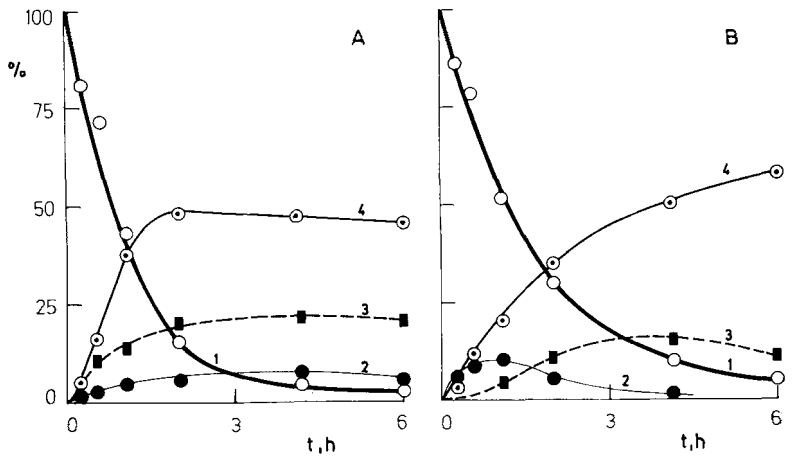

FIG. 4. The effect of different concentrations of ATP on the time course of cytidine-^{14}C(U) phosphorylation by a cell-free extract of mouse leukemic cells.

Incubations were carried out as in Figure 1 using 0.1 ml of an enzyme fraction. 1×10^{-4}M cytidine-2-^{14}C, 2.5×10^{-3}M Mg^{2+}-ions and 2.5×10^{-3}M (A) and 5×10^{-3}M (B) adenosine-5'-triphosphate, resp. 1, Cytidine; 2, cytidine-5'-monophosphate; 3, cytidine-5'-diphosphate; and 4, cytidine-5'-triphosphate as per cent of the total radioactivity (20 μCi).

TABLE 2. Transformations of cytidine-^{14}C(U) in a cell-free extract from mouse leukemic cells.

Incubation was carried out in 2×10^{-2}M Tris-HCl buffer, pH 8.1 at 37° C in a total volume of 1 ml with A – 0.1 ml and B – 0.3 ml, resp., of an enzyme fraction (sonication 10 sec; centrifugation 2 h at 110,000xg). 1×10^{-4}M cytidine-2-^{14}C and 2.5×10^{-3}M adenosine-5′-triphosphate with equimolar Mg^{2+}-ions. Radioactivity of the compounds is expressed as % of the total radioactivity of the reaction mixture.

	Incubation time (min)							
	A :				B :			
Compound	30	60	120	240	30	60	120	240
CR-^{14}C	71.7	30.1	13.0	7.7	31.0	9.5	8.7	6.7
5′-CMP-^{14}C	3.1	3.1	1.5	5.2	3.8	4.8	5.7	18.7
5′-CDP-^{14}C	7.1	9.9	17.7	16.9	10.7	14.4	14.7	14.1
5′-CTP-^{14}C	10.6	40.0	41.0	48.3	29.1	38.2	30.9	11.8
UR-^{14}C	7.5	20.3	17.4	11.4	13.6	7.4	2.6	4.6
5′-UMP-^{14}C	—	1.1	2.9	4.1	6.7	13.9	20.8	29.3
5′-UTP-^{14}C	—	—	6.6	6.7	5.1	12.3	17.4	16.7

Purine nucleosides are not suitable substrates for the synthesis of radioactive 5′-nucleotides in the system under study. The number of different products observed in this system using guanosine-^{14}C as substrate is so considerable that no reliable chromatographic or electrophoretic identification could be made. At the same time the yield of guanine-5′-phosphates was minimal.

From the viewpoint of possible technological application the phosphorylation of adenosine-^{14}C is more promising. During the initial stage of phosphorylation (Table 3) a pronounced deamination of adenosine-^{14}C to inosine-^{14}C takes place; thereafter phosphorylation of inosine-^{14}C to inosine-5′-diphosphate-^{14}C is observed. At the endpoint of incubation inosine-5′-phosphates represent almost 50 % of the total radioactivity.

C. — *Synthesis of uridine-5′-nucleotides-^{14}C(U) on the semi-preparative scale.*

(a) *Synthesis of uridine-5′-monophosphate-^{14}C(U).*

Uridine-^{14}C(U) (220 μCi; 1 μmole; 246 μg), adenosine-5′-triphosphate (25 μmoles); Mg^{2+}-ions (25 μmoles); creatine phosphate (25 mg) and creatine

TABLE 3. Products formed during the phosphorylation of adenosine-^{14}C(U) by a cell-free extract from mouse leukemic cells.

Incubation was carried out in 2×10^{-1}M Tris-HCl buffer, pH 8.1 at 37° C in a total volume of 1 ml with 0.1 ml of an enzyme fraction (sonication 10 sec; centrifugation 2 h at 110,000 g). 1×10^{-4}M adenosine-^{14}C(U) and 2.5×10^{-3}M adenosine-5′-triphosphate with equimolar Mg^{2+}-ions. Radioactivity of the compounds is expressed in % of the total radioactivity of the reaction mixture.

Compound	Incubation time (min)				
	1	5	15	30	120
AR-^{14}C	56.5	1.0	1.4	1.2	0.1
I-^{14}C	40.3	73.5	66.6	44.5	12.3
5′-IMP-^{14}C	3.2	25.5	29.4	32.7	14.7
5′-IDP-^{14}C	0	0	0	8.8	31.7
Total	100	100	97.4	87.2	58.8

kinase (0.8 mg) were dissolved in 3 ml of 0.02 M Tris-HCl buffer (pH 8.1) and incubated for 2 h at 37° C with 1.0 ml of fresh enzyme from mouse leukemic livers (60 sec of sonication at the maximum output of the ultrasonic disintegration MSE, 50,000xg, 1 h). Incubation was interrupted by the addition of 4 ml of 96 % ethanol, and the sample was kept at 3° C for 2 hr. The precipitate was discarded by centrifugation (5,000xg, 20 min). The volume of the supernatant fraction was reduced by distillation in vacuo at 40° C and chromatographed on a Whatman paper No. 3 (isobutyric acid — water — ammonium hydroxide).

After drying the chromatogram in the stream of cold air autoradiography was carried out (18 hr). The strips of paper corresponding to uridine-5′-tri- and -monophosphate-^{14}C(U), uridine-^{14}C(U), and uracil-^{14}C(U) were cut out, washed with n-butanol saturated with water and the radioactive substances were eluted with ammonium hydroxide-ethanol (1 : 1)-UTP-^{14}C(U)- or with water. Total radioactivity was determined in the eluates and radiochemical purity of uridine-5′-monophosphate-^{14}C(U) was assayed. Total yield was 128 μCi of uridine-5′-monophosphate-^{14}C(U) of radiochemical purity higher than 98 % (58 % of the initial radioactivity). At the same time 20 μCi of uridine-^{14}C was obtained (9.1 % of the initial radioactivity).

(b) *Synthesis of uridine-5′-diphosphate-^{14}C(U).*

Uridine-5′-diphosphate-^{14}C(U) was prepared as described [12]. Ethanolic solution of uridine-5′-triphosphate-^{14}C(U) (110 μCi, 0.5 μmole) was evapo-

rated to dryness. And subsequently dissolved in 0.3 ml of 0.1 N-HCl and hydrolysed in a sealed ampoule at 55-60° C for 4 h. Hydrochloric acid was discarded by freeze-drying, the residue was dissolved in the minimal volume of 50 % ethanol solution, and chromatographed as described. The total yield was 62 μCi (56.3 % of the initial radioactivity) of uridine-5'-diphosphate-^{14}C(U), its radiochemical purity exceeded 96 %. Besides the main product uridine-5'-mono- and triphosphate-^{14}C(U) (24.5 % and 9.1 %, respectively of the initial radioactivity) were isolated.

(c) *Synthesis of uridine-5'-triphosphate-^{14}C(U).*

Uridine-^{14}C(U) (220 μCi; 1 μmole), Mg^{2+}-ions (25 μmoles), adenosine-5'-triphosphate (25 μmoles), 1 ml of enzymatic preparation (sonication 10 sec at the maximal output of the ultrasonic disintegrator MSE, 110,000xg, 2 h) in 0.02M Tris-HCl buffer (pH 8.1) were incubated at 37° C for 240 min (total volume 5 ml). Further procedure has already been described. Total yield was 144 μCi of uridine-5'-triphosphate-^{14}C(U), radiochemical purity exceeded 97 % (66 % of the initial radioactivity).

V. — Conclusions

The enzymatic phosphorylation of radioactive nucleosides in the presence of cell-free extract from leukemic mouse livers is generally useful for the production of different radioactive 5'-nucleotides. The method is especially suitable for the preparation of radioactive pyrimidine 5'-nucleotides. Somewhat lower formation of cytosine-^{14}C 5'-nucleotides is due to the presence of deaminases. The phosphorylation of adenosine-^{14}C is accompanied by its complete deamination to inosine-^{14}C during the initial stage of incubation. Inosine-^{14}C is phosphorylated to hypoxanthine 5'-nucleotides. The phosphorylation of guanosine-^{14}C by the present method is not convenient.

The described procedure has many advantages : Unpurified cell-free extract from leukemic cells may be applied directly. The synthesis of radioactive 5'-nucleotides may be controlled to obtain mono-, di-, or triphosphates. This requires suitable concentration of the enzyme and of adenosine-5'-triphosphate in the reaction mixture. If the increased level of adenosine-5'-triphosphate in the reaction mixture is not employed the application of the ATP-regenerating system may be of value. Furthermore, the nucleotide synthesis may be controlled by the different treatments of leukemic cell homogenate. The duration of sonication and the centrifugal forces applied while spinning down the sonicate are of major significance.

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