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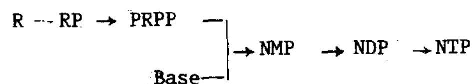
ENZYMATIC SYNTHESIS OF MULTIPLE TRITIUM-LABELED NUCLEOSIDES AND NUCLEOTIDES FROM NITROGEN BASES AND RIBOSE

E. V. Snetkova, Yu. L. Kaminskii,
G. P. Akulov, and Z. K. Nikolaeva

UDC 577.113.3:546.11.3

A wide range of highly active nucleotides containing tritium labels in the heterocycle nucleus and ribose residue has been synthesized with the aid of an enzyme preparation from *E. coli*, starting from tritium labeled nitrogen bases and ribose. In individual cases, the complex enzyme preparation was modified by the addition of nucleotide kinases or phosphatases.

Recently, considerably interest has been aroused by the biosynthesis of nucleotides from nitrogen bases with elimination of the stage of forming nucleosides. Analogous reactions in vitro provide the possibility of synthesizing nucleoside 5'-triphosphates (NTPs) from bases and 5-phosphoribosyl 1-pyrophosphate (PRPP) without the isolation of the intermediate products; the phosphoribosyl pyrophosphate can be replaced by its precursors — ribose 5-phosphate (RP) or ribose (R):



The complex (multifunctional) enzyme preparation used must contain enzymes catalyzing the conversion of ribose into ribose 5-phosphate (ribokinase, EC 2.7.1.15), ribose 5-phosphate into phosphoribosyl pyrophosphate (ribose phosphate pyrophosphokinase, EC 2.7.6.1), phosphoribosyl pyrophosphate and nitrogen bases into nucleoside 5'-monophosphates (adenine phosphoribosyltransferase, EC 2.4.2.7; uracyl phosphoribosyltransferase, EC 2.4.2.9, etc.); and nucleoside 5'-monophosphates into the corresponding di- and then triphosphates (specific nucleoside 5'-monophosphate kinases, EC 2.7.4, and nonspecific nucleoside 5'-diphosphate kinase, EC 2.7.4.6). This set of enzymes is present in various cultures — *E. coli* [2-8], *Saccharomyces cerevisiae* [9, 10], *Brevibacterium ammoniagenes* [11, 12], *Corynebacterium species* [13], etc. Depending on the ratio of the activities of the individual enzymes in the preparation used and the nature of the nitrogen base, the predominating reaction product consists of nucleoside 5'-monophosphates or nucleoside 5'-triphosphates. In order to increase the yield of nucleoside 5'-triphosphates, the activity of the ATP-regenerating system is usually enhanced by the supplementary addition to the incubation mixture of creatine phosphate and creatine phosphokinase (EC 2.7.3.2) or phosphoenol pyruvate and pyruvate kinase (EC 2.7.1.40) [12].

As applied to the synthesis of labeled nucleotides, the majority of investigations have been limited to the use of phosphoribosyl pyrophosphate [5, 6, 9-10, 12] and to the presence

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V. G. Khlopin Radium Institute and Biological Scientific Research Institute, A. A. Zhdanov Leningrad State University. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 258-264, March-April, 1988. Original article submitted April 11, 1987.

0009-3130/88/2402-0221$12.50 © 1988 Plenum Publishing Corporation

of a tritium or carbon label only in the nitrogen bases [2-12]. Such limitations were dictated by the unavailability of labeled phosphoribosyl pyrophosphate; in addition, the enzymes ensuring its formation are the least stable and are the first to be inactivated in the process of isolating a complex enzyme preparation [3, 10].

As a rule, no information whatever is given on the molar activity (A_{mol}) of labeled nucleotides [2-5, 1, 13]. In those few cases where the A_{mol} values of the initial bases and the final nucleotides are reported or the question of the retention of the level of the label in the course of the reaction is discussed without specifying A_{mol}, the statements are frequently contradictory. Thus, in [6, 7] the retention of A_{mol} in the synthesis of [2,8-^{3}H]-ATP and of [5,6-^{3}H]UTP is reported, although in [8] it is stated to decrease in the synthesis of [5,6-^{3}H]UTP on working with the same enzyme preparation. There are examples of almost twofold differences in A_{mol} of the 5'-mono- and 5'-triphosphates of the same nucleoside isolated from one and the same incubation mixture [12].

Our task was to synthesize from nitrogen bases and ribose a wide range of highly active nucleotides and nucleosides containing a multiple tritium label in the heterocyclic and carbohydrate moieties.

Initial experiments on the synthesis of [^{3}H]ATP and [^{3}H]UTP were performed with the complex enzyme preparation 1A obtained in [3] under the conditions there recommended. It was found here that the values of A_{mol} for the [^{3}H]ATP and [^{3}H]UTP isolated were 2-6 times lower than the A_{mol} values of the initial [^{3}H]adenine and [^{3}H]uracil (or the sum of the A_{mol} values of the corresponding base and [^{3}H]ribose. Furthermore, the spectral characteristics of the [^{3}H]NTPs differed appreciably from the corresponding magnitudes for nonradioactive compounds. In syntheses performed with bases containing a double label, the ^{3}H/^{14}C ratio in the final NTPs and the initial bases coincided. Radiochromatographic analysis showed the possibility of the formation of [^{3}H]ATP from [^{3}H]ribose even when no [^{3}H]adenine and ATP were added to the incubation mixture (se below). It follows from this that the reason for the low A_{mol} values of [^{3}H]NTPs in comparison with the A_{mol} values of the initial [^{3}H]-bases is foreign nucleotide material present in the inadequately purified complex enzyme preparation.

In actual fact, solutions of this preparation had an optical density of about 4 OU_{260} (per 1 mg of protein), while for an average protein this magnitude corresponds to ~1 [14]. "Blank" experiments on the chromatographic separation of incubation mixtures to which nitrogen bases and ATP had not been specially added showed a considerably amount of various UV-absorbing impurities having retention volumes close to the retention volumes of NMPs and NTPs.

Another disadvantage of complex enzyme preparation 1A is the necessity for adding additional amounts of nonradioactive ATP (up to 200% in relation to the initial base) for the incubation mixture, which, as applied to the use of [^{3}H]adenine likewise made the synthesis of a preparation with a high value of A_{mol} impossible.

We therefore subsequently renounced the use of the complex enzyme preparation 1A and took as a basis the method of isolating and purifying a similar preparation developed by Myasoedov et al. [7, 8]. The main distinguishing feature of this method consists in the elimination of nucleotide material by the treatment of the preparation with DNase and RNase followed by dialysis (the preparation obtained had an optical density of 0.15 OU_{457} per 1 mg of protein). Furthermore, the high level of enzymatic activity in the preparation permitted a substantial lowering of the amount of ATP added to the incubation mixture, to only 10% in relation to the [^{3}H]adenine. Certain changes that we introduced into this method relating to the strain of E. coli used the phases of growth of the cells, details of isolation, and also the production of the material in the freeze-dried state (preparation 1B; see the Experimental part); it was possible to lower the optical density of the preparation to 0.06-0.09 OU_{257}/mg of protein.

It was found that of bases of the purine series the most active in the enzymatic synthesis of NTPs were adenine and guanine (Fig. 1). The formation of [2,5',8-^{3}H]ATP was complete after 1 h with a yield of about 95%. After 5 h, a small amount (1.6%) of [2,5',8-^{3}H]adenosine appeared, and after a day this still did not exceed 8%. The conversion of guanine took place similarly and was complete after 1.5 h with a yield of [5',8-^{3}H]GTP of about 85%; no appreciable amounts of [5',8-^{3}H]guanosine were observed even after a day.

To obtain [2,5'8-^{3}H] adenosine and [5',8-^{3}H]guanosine, an immobilized preparation of alkaline phosphatase (II) was added to the incubation mixtures before the maximum yield of the

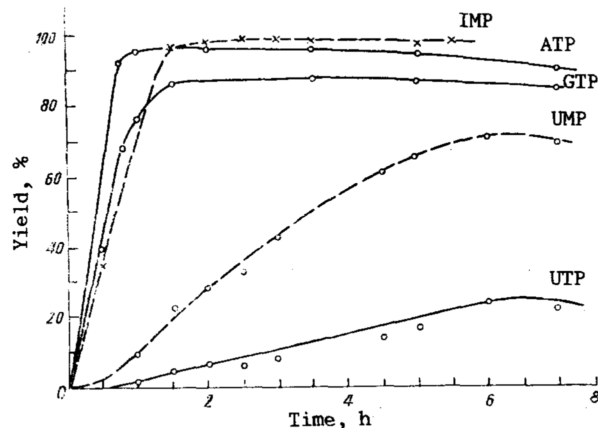

Fig. 1. Kinetics of the synthesis of nucleotides from [^{3}H]bases and ribose.

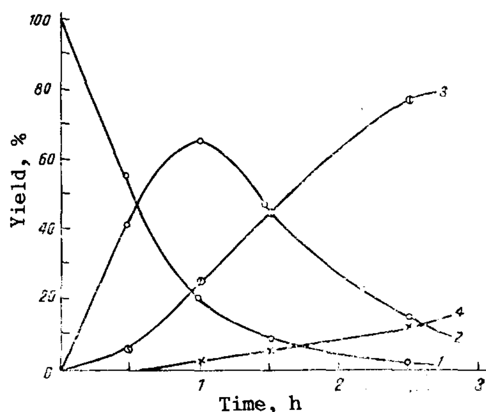

Fig. 2. Kinetics of the change in the composition of the incubation mixture in the enzymatic reaction of [5-^{3}H]cytosine and ribose: 1) [5-^{3}H]cytosine; 2) [5-^{3}H]uracil; 3) [5-^{3}H]UMP; 4) [5-^{3}H]UTP.

NTPs had been reached. After incubation for 1 h, the amount of [2,5',8-^{3}H]adenosine and that of [5',8-^{3}H]guanosine were 35%.

With [2,8-^{3}H]hypoxanthine, the reaction took place fairly rapidly but its main product was [2,5',8-^{3}H]INP, which was formed with a yield of more than 95% after 1.5 h (Fig. 1). Double the amount of enzyme in comparison with that normally used (see the Experimental part) led exclusively to [2,5',8-^{3}H]inosine. These facts indicate the absence of IMP kinase activity in the complex enzyme preparation.

In the reaction of [5,6-^{3}H]uracil, a comparatively slow accumulation of [5,5',6-^{3}H]UMP was observed, its maximum amount (about 70%) being recorded after incubation for 6 h (see Fig. 1). Doubling the amount of enzyme preparation accelerated the transformation of the [5,5',6-^{3}H]UMP in two opposite directions, increasing the yield of [5,5',6-^{3}H]UTP to 40% and that of [5,5',6-^{3}H]uridine to 20%. Therefore to achieve an acceptable yield of [5,5',6-'H]UTP after the completion of a six-hour incubation a preparation of nucleotide kinase (III) was also added to the mixture and incubation was continued for another 1 h, which led to the formation of [5,5',6-^{3}H]UTP with a yield of about 70%. A similar approach can be applied to the synthesis of higher nucleoside phosphates from [2,8-^{3}H]hypoxanthine.

The predominating transformation of [5-^{3}H]cytosine under the action of the complex enzyme preparation is deamination, as a result of which even in the first 30 minutes of incubation the amount of [5-^{3}H]cytosine and that of the [5-^{3}]uracil formed from it becomes comparable (Fig. 2). Only the [5-^{3}H]uracil takes part in further transformations, since nucleosides and nucleotides of the uracil series, alone, were detected in the reaction products. Apparently, a report of the formation of [5-^{3}]CTP from [5-^{3}H]cytosine and ribose [3, 4], was erroneous, since it contradicts the results of our work and does not agree with available information [15] on the absence of cytosine phosphoribosyltransferase activity in *E. coli* cultures.

TABLE 1. Molar Activity and the Products of Enzymatic Syntheses

Reactant	A_{mol}, TBq/mole (kCi/mole)	Reactant	Mean A_{mol}, TBq/mole (KCi/mole)
[2,8-^{3}H] Adenine [5-^{3}H] Ribose	1550 (42) 670 (18)	[2,5', 8-^{3}H] ATP [2,5', 8-^{3}H] Adenosine	2100 (57) 2100 (57)
[2,8-^{3}H] Adenine Ribose	1550 (42)	[2,8-^{3}H]ATP [2,8-^{3}H]Adenosine	1520 (41) 1520 (41)
[8-^{3}H] Guanine [5-^{3}H] Ribose	890 (24) 670 (18)	[5', 8-^{3}H]GTP [5', 8-^{3}H] Guanosine	1440 (39) 1440 (39)
[8-^{3}H] Guanine Ribose	890 (24)	[8-^{3}H] GTP [8-^{3}H]Guanosine	850 (23) 850 (23)
[2,8-^{3}H] Hypoxanthine [5-^{3}H] Ribose	1220 (33) 670 (18)	[2,5', 8-^{3}H] Inosine	1850 (50)
[5,6-^{3}H]Uracil [5-^{3}H] Ribose	1850 (50) 670 (18)	[5,5', 6-^{3}H] UTP [5,5', 6-^{3}H]Uridine	2520 (68) 2520 (68)
[5,6-^{3}H]Uracil Ribose	1850 (50)	[5,6-^{3}H] UTP [5,6-^{3}H] Uridine	1740 (47) 1740 (47)
[5-^{3}H] Cytosine Ribose	960 (26)	[5-^{3}H] UTP	930 (25)

It is interesting to note that the formation of [5-^{3}H]UMP (A_{mol} = 930 TBq/mole from [5-^{3}H]cytosine and ribose takes place approximately twice as fast as the formation of [5,5', 6-^{3}H]UMP (A_{mol} = 2520 TBq/mole) from [5,6-^{3}H]uracil and [5-^{3}H]ribose (compare Figs. 1 and 2). It may be assumed that the reason for this consists in the partial inactivation of the enzymes responsible for the synthesis of UMP under the influence of trace impurities present in the tritium substrates with high A_{mol} values. We have observed a similar phenomenon previously in the enzymatic synthesis of multiple tritium-labeled thymidine [16].

All the enzymatic transformations performed with preparation 1B were characterized by their retention of the tritium label: the A_{mol} values of the nucleosides and nucleotides obtained were close to the sums of the A_{mol} values of the [^{3}H]bases and [5-^{3}H]ribose used in the synthesis (Table 1). The spectral characteristics of the compounds synthesized corresponded to literature figures for the unlabeled analogs.

Thus, in the present work we have effected the synthesis of nucleosides and nucleotides containing a tritium label in the heterocyclic nucleus and in the ribose residue, with molar activities of up to 2590 TBq/mole (70 kCi/mole) with the aid of a complex enzyme preparation from E. coli modified where necessary by the addition of phosphatases or nucleotide kinases.

EXPERIMENTAL

Enzyme Preparations from E. coli Possessing Phosphoribosyl-Pyrophosphate- and Nucleoside-Triphosphate-Synthesizing Activity (I). 1A. A preparation isolated from E. coli AB 259 Hfr 3000 thi as in [3] was used (provided by the Institute of Organic Synthesis of the LatvSSR Academy of Sciences).

1B. The E. coli strain His B 463 was grown in complete nutrient medium [17] to the middle of the logarithmic growth phase.

The cells were separated by centrifugation and were twice washed with 0.15 M NaCl. A solution of lysozyme was added to the suspended moist cells in a ratio of 1 mg of lysozyme in 0.3 ml of water to 1 g of cells. The dense suspension was subjected to rapid freezing at −70°C and thawing at 4°C. Then 0.05 M potassium phosphate buffer, pH 7.4, containing EDTA and 2-mercaptoethanol were added over 30 min to give final concentrations of 0.1 and 7 mM, respectively (2 ml of buffer per 1 g of moist cells). To precipitate the nucleic acids, 10% streptomycin sulfate was added (0.6 ml of solution per 1 g of E. coli cells). After 30 min, the nucleic acid that had precipitated and the cell residues were eliminated by centrifugation at 18,000 rpm (~40,000 × g) for 60 min.

After the supernatant had been dialyzed against 0.05 M potassium phosphate buffer, pH 7.4, with 7 mM 2-mercaptoethanol for 6 hours, deoxyribonuclease and ribonuclease were added to it to give final concentrations of 5 and 300 μg/ml, respectively, and also $MgCl_2$ to a concentration of 5 mM, and the solution was incubated at 37°C for 75 min. The precipitate that deposited was eliminated by centrifugation at 18,000 rpm for 10 min.

The supernatant was dialyzed against the same buffer for 16 h, after which the protein fraction was precipitated by the addition of solid ammonium sulfate to 60% saturation at pH 7.0. The precipitate was dissolved in phosphate buffer containing 2-mercaptoethanol and was dialyzed for 24 h, after which the solution was lyophilized. In this form, the enzyme preparation can be stored at 4°C for not less than a year.

The residual nucleotide material amounted to 0.06-0.09 OU_{257}/mg of protein. The specific activity of the preparation was 0.3 unit/mg (as the unit of activity we took the amount of enzyme preparation converting 1 μmole of adenine into ATP in 30 min).

Other Enzymes. We used creating phosphokinase from Reanal; deoxyribonuclease from the FRG; ribonuclease from the USA; and *E. coli* B alkaline phosphatase (II) immobilized on Sepharose with a specific activity of 4.9 units/mg of moist preparation; and also a preparation of nucleotide kinases (III) from *E. coli* B infected with phage T4am 82, with a specific activity of 0.32 unit/mg.

Labeled Compounds. We used [2,8-^{3}H]adenine with A_{mol} 1550 TBq/mole (42 kCi/mole), [8-^{3}H]guanine with A_{mol} 890 TBq/mole (24 kCi/mole), [2,8-^{3}H]hypoxanthine with A_{mol} 1220 TBq/mole (33 kCi/mole), [5,6-^{3}H]uracil with A_{mol} 1850 TBq/mole (50 kCi/mole), [5-^{3}H]cytosine with A_{mol} 960 TBq/mole (26 kCi/mole), and [5-^{3}H]ribose with A_{mol} 670 TBq/mole (18 kCi/mole) synthesized in the V. G. Khlopin Radium Institute, and also [1-^{14}C]ribose, [8-^{14}C]adenine, and [2-^{14}C]-uracil supplied by the Izotop V/O [All-Union Amalgamation].

Synthesis of Nucleotides. The incubation mixture in the synthesis of the nucleotides contained 1 μmole of [^{3}H]base, 2 mmole of [5-^{3}H]ribose, 50 μmole of creatine phosphate, 10 μmole of $MgCl_2$, 0.1 μmole of ATP, 0.5 mg of creating phosphokinase, and 4 mg of enzyme preparation 1B. Incubation was performed in potassium phosphate buffer, pH 7.9, 125 μmole (the use of Tris buffer with the same pH value lowered the rate of the reaction to some extent) at 37°C. The volume of the incubation mixture was 1.25 ml. The optimum time of incubation (between 1 and 6 h) was determined on the basis of kinetic investigations in each concrete case.

If the main product of the synthesis was a NMP, before the maximum yield of the latter in the incubation mixture had been reached 1-2 activity units of nucleotide kinase was added to the incubation mixture and incubation was continued for another 1-2 h in order to obtain higher phosphates (as the unit of activity of the nucleotide kinase preparation we took that amount of it which led to the conversion of 10 nmole of TMP into TTP in 30 min).

The reaction was stopped by the addition of 2 ml of ethanol to the incubation mixture followed by heating in the boiling water bath for 1 min. The coagulated protein was filtered off, and the filtrate was deposited on a 7 × 250 mm column of DEAE-Sephadex A-25 (OH^-). Elution was performed with water (60 ml) and then with bicarbonate buffer (pH 8.65) in a linear concentration gradient created by mixing 150 ml of water and 150 ml of 0.5 M NH_4HCO_3 at the rate of 0.96 ml/min. The process of chromatography was monitored from the electrical conductivity of the eluate and its absorption in the UV region, and also from its radioactivity.

To eliminate bicarbonate, to a portion of the eluate containing a NTP was added SE-sephadex C-25 (H^+) to pH 6, after which the pH was brought to 7 with a solution of ammonia, and the Sephadex was filtered off and was washed with water (2 × 3 ml).

Synthesis of Nucleosides. [2,5',8-^{3}H]Adenosine and [5',8-^{3}H]guanosine were obtained by adding about 50 activity units of alkaline phosphatase to the initial incubation mixture before the maximum yield of NTP had been reached and incubating for another 1 h. (As the unit of activity of the alkaline phosphatase we took the amount of enzyme causing the liberation of 1 μmole of p-nitrophenol in 1 h at 37°C in a 0.02 M solution of p-nitrophenyl phosphate).

The desalting and purification of nucleosides were carried out on a 18 × 650 mm column of Sephadex G-10 with elution by water at the rate of 0.96 ml/min.

The compositions of the reaction mixtures and the radiochemical purities of the preparation were determined by thin-layer radiochromatography on Silufol in the dioxane—ammonia—water (6:1:4) solvent system.

SUMMARY

Syntheses of various highly active nucleoside triphosphates from tritium-labeled bases and ribose have been performed with the aid of a complex enzyme preparation. When the incu-

bation mixtures were treated additionally with alkaline phosphates it was possible to convert the nucleotides obtained into the corresponding multiple labeled nucleosides.

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