New Possibilities of Enzyme Synthesis of Radioactive Nucleotides.

II. Phosphoribosylation of radioactive bases of nucleic acids by the catalytic effect of unpurified cell-free extract of *Brevibacterium ammoniagenes*

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SUMMARY

The catalytic properties of an unpurified enzyme preparation from cells of Brevibacterium ammoniagenes *toward phosphoribosylation of the individual nucleic acid bases are pronouncedly specific, display a high efficiency toward phosphoribosylation of uracil where the conversion to uridine-5'-phosphate is quantitative, phosphoribosylation of adenine takes place with lower efficiency and guanine and cytosine are practically not phosphoribosylated at all. Using orotic acid-^{14}C and a complete inhibition of orotidine 5'-phosphate decarboxylase by means of 6-azauridine-5'-phosphate, a 60 % production of orotidine-5'-phosphate-^{14}C has been achieved. The catalytic efficiency of the enzyme preparation remains preserved even upon substitution of 5-phosphoribosyl-1-pyrophosphate by its direct metabolic precursors.*

The possibility of direct application of the unpurified cell-free extract of the bacteria, the simple composition of the final reaction mixtures and the high degree of production of biologically important compounds makes it possible to employ the method studied for an enzyme synthesis of radioactive uridine-5'-phosphate, orotidine-5'-phosphate and adenosine-5'-phosphate.

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INTRODUCTION.

During study of the pyrimidine pathway of *Brevibacterium ammoniagenes* and of its mutants it was found [1] that practically the only anabolite formed from orotic acid and 5-phosphoribosyl-1-pyrophosphate in the presence of unpurified bacterial extracts is uridine-5′-phosphate. On the other hand, in the presence of 6-azauridine-5′-phosphate which inhibits specifically orotidine-5′-phosphate decarboxylase [2] the enzyme system displays a remarkable conversion of orotic acid to orotidine-5′-phosphate.

Proceeding from these fundamental findings we decided to make use of the specific properties of bacterial extracts for developing an enzyme synthesis of uridine-5′-phosphate and orotidine-5′-phosphate labelled specifically with ^{14}C, using orotic acid-6-^{14}C as substrate.

In the present communication the attention is concentrated on the investigation of the specificity of phosphoribosylation of the purine and pyrimidine bases of nucleic acids in the presence of unpurified bacterial extracts of *Brevibacterium ammoniagenes*. In a broader context, especially in the case of phosphoribosylation of uracil-^{14}C, the problem is examined from the point of view of possible effective radioactive synthesis of uridine-5′-phosphate-^{14}C. We thought it also useful to analyze in greater detail the enzyme synthesis of radioactive orotidine-5′-phosphate-^{14}C under conditions of total inhibition of orotidine-5′-phosphate decarboxylase with the aid of 6-azauridine-5′-phosphate.

MATERIALS.

Uracil-2-^{14}C and cytosine-2-^{14}C of specific activity 44 mCi/mmole, adenine-8-^{14}C of specific activity 25 mCi/mmole and guanine-^{14}C(U) of specific activity 130 mCi/mmole, were the products of the Institute for Research, Production and Uses of Radioisotopes in Prague. Orotic acid-6-^{14}C of specific activity 24.3 mCi/mmole was a product of Calbiochem (USA). All the radioactive compounds are designated as radiochemically pure. 5-Phosphoribosyl-1-pyrophosphate (dimagnesium, dihydrate), adenosine-5′-triphosphate (disodium, trihydrate) and ribose-5′-phosphate (sodium salt, sesquihydrate) were also products of Calbiochem (USA).

METHODS.

Selected chromatographic systems suited for separation of products of the studied enzyme reactions were applied. The optimum separation capacity was displayed by paper chromatography in isobutyric acid : water : concentrated ammonia (66 : 33 : 1.5) [4]. Development in this system was done on Whatman No. 3 at room temperature for 20 h in the descending direction. Radioactive compounds on the chromatograms were detected autoradio-

graphically on Agfa X-ray films while the reference standards of nucleic acid components were identified as spots quenching the UV light of a Chromatolite lamp. The chromatograms were evaluated quantitatively by counting them under a β-tube of the chromatogram scanner of Frieseke-Hoepfner and by planimetry of the record obtained. Radioactivity of the aqueous solutions of compounds labelled with ^{14}C was determined with the aid of an end-window GM counter of defined geometric angle. The liquid investigated was in a saturated layer with respect to the radionuclide ^{14}C and calibration was done in the same geometry with the aid of the ER-2 ^{14}C standard (product of the Institute for Research, Production and Uses of Radioisotopes in Prague).

Results.

Preparation of the cell-free extract of Brevibacterium ammoniagenes.

The *Brevibacterium ammoniagenes* strain CCEB 364 was grown on meat-peptone broth with phosphate [5] of pH 7.2, under aerobic conditions on a reciprocal shaker at 30° C for 24 h. 500 ml of the bacterial suspension was centrifuged under cooling and the sediment was washed with 250 ml physiological saline. A total of 5 g wet weight of bacteria was obtained. The bacteria were suspended in 20 ml 0.02 M Tris-HCl buffer of pH 7.4 and the suspension was sonicated for 5 min under cooling in a MSE 60 W disintegrator. Centrifugation in the cold (3,000 g, 60 min) yielded a clear supernatant. Lowry's method [6] displayed in 1 ml suspension a total of 13 mg protein. The preparation was stored in smaller lots at −20° C.

Time course of the phosphoribosylation of uracil-^{14}C catalyzed by the cell-free extract of Brevibacterium ammoniagenes.

A mixture of 0.1 ml 2.10^{-1}M Tris-HCl buffer of pH 7.4, 0.1 ml 2.10^{-2}M $MgSO_4$, 0.2 ml 10^{-2}M 5-phosphoribosyl-1-pyrophosphate, 0.1 ml 6.10^{-3}M uracil-2-^{14}C and 0.1 ml enzyme made with distilled water to 1 ml, was incubated at 30° C. In the course of the reaction, 0.02 ml samples were removed from the reaction mixture at suitable intervals and analyzed immediately by paper chromatography. The time course of phosphoribosylation of uracil-2-^{14}C follows from Table 1.

The composition of the starting mixture as well as the other reaction conditions selected for the above case of phosphoribosylation are considered as standard. Under these standard conditions an equilibrium is reached during three hours of the reaction when, at the same time, the conversion of uracil-2-^{14}C to uridine-5′-phosphate-2-^{14}C is quantitative and the uridine-5′-phosphate is the sole reaction product. For several further hours no changes in the composition of the reaction mixture are observed. Only during the later reaction phases cleavage back to uracil-2-^{14}C takes place, as well as the

TABLE 1. Time course of phosphoribosylation of uracil-2-^{14}C catalyzed by the cell-free extract of *Brevibacterium ammoniagenes* [a] (Radioactivity of the compounds is given in % of total ^{14}C radioactivity).

Compound	R_f	Reaction period (h)												
		0	1	2	3	4	5	6	8	10	22	28	46	52
Start	0.00	—	—	—	—	—	—	—	1.0	1.6	5.2	5.9	9.2	10.4
Uridine-5′-phosphate-2-^{14}C	0.32	—	74.0	90.0	100	100	100	100	99.0	98.4	84.2	81.3	73.6	70.3
Uracil-2-^{14}C	0.63	100	26.0	10.0	—	—	—	—	—	—	10.6	12.8	17.1	19.4

[a] Reaction conditions are given in the text.

Table 2. Time course of phosphoribosylation of adenine-8-^{14}C catalyzed by the cell-free extract of *Brevibacterium ammoniagenes* [a]. (Radioactivity of the compounds is given in % of total ^{14}C radioactivity).

Compound	R_f	Reaction period (h)											
		0	1	2	3	4	5	6	8	10	22	28	48
Start	0.00	0.9	1.0	1.2	1.5	1.3	2.0	2.6	2.8	3.3	14.3	19.7	34.4
Adenosine-5′-phosphate-8-^{14}C	0.59	1.0	1.5	3.8	5.6	6.6	11.7	13.1	15.4	18.4	29.4	32.2	6.8
Hypoxanthine-8-^{14}C	0.65	—	—	—	—	—	—	—	—	4.6	20.0	24.2	26.7
Adenosine-8-^{14}C	0.76	—	—	—	—	—	—	—	—	—	4.8	12.2	29.8
Adenine-8-^{14}C	0.89	98.1	97.5	95.1	93.0	92.3	86.0	84.3	81.8	74.0	31.5	11.7	2.3

[a] Reaction conditions are given in the text.

formation of another radioactive compound in the reaction mixture which, in the given chromatographic system, has zero mobility. The identification of this compound represents the object of further research.

Study of phosphoribosylation of ^{14}C-adenine, guanine and cytosine, catalyzed by the cell-free extract of Brevibacterium ammoniagenes.

Using the conditions described for uracil-2-^{14}C, experiments with phosphoribosylation of adenine-8-^{14}C, guanine-^{14}C(U) and cytosine-2-^{14}C were carried out.

Table 2 shows that phosphoribosylation of adenine-8-^{14}C proceeds at a much lower rate than the phosphoribosylation of uracil-2-^{14}C, at a maximum of 40 % conversion of adenine-8-^{14}C to adenosine-5'-monophosphate-8-^{14}C during 22 hours of incubation period. During the later phase enzymatic dephosphorylation of adenosine-5'-phosphate to adenosine-8-^{14}C as well as a deamination of adenine-8-^{14}C to hypoxanthine-8-^{14}C take place. Much more pronounced during the later incubation phase is the accumulation of an unidentified ^{14}C-labelled compound at the radiochromatogram start.

The cell-free extract of *Brevibacterium ammoniagenes* does not catalyze the phosphoribosylation of cytosine-2-^{14}C. On the other hand (see Table 3), one can record in the course of the reaction an increasing deamination of cytosine-2-^{14}C to uracil-2-^{14}C and again a pronounced accumulation of a radioactive compound at the radiochromatogram start. Similarly, guanine-^{14}C(U) does not undergo any striking changes in the enzyme system investigated.

Possibilities of substitution of 5-phosphoribosyl-1-pyrophosphate with its metabolic precursors during synthesis of uridine-5'-monophosphate-^{14}C in the presence of the enzyme from Brevibacterium ammoniagenes.

The possibility of substituting the immediate metabolic precursors, adenosine-5'-triphosphate and ribose-5-phosphate, for 5-phosphoribosyl-1-pyrophosphate during the synthesis of uridine-5'-monophosphate-2-^{14}C from uracil-2-^{14}C was studied in the presence of the cell-free extract of *Brevibacterium ammoniagenes*. In this case, a mixture of 0.1 ml 2.10^{-1}M Tris-HCl buffer of pH 7.4, 0.1 ml 2.10^{-2}M $MgSO_4$, 0.1 ml 6.10^{-3}M uracil-2-^{14}C, 0.1 ml 6.10^{-2}M adenosine-5'-triphosphate, 0.1 ml 6.10^{-2}M ribose-5-phosphate and 0.1 ml enzyme, made with distilled water to the total volume of 1 ml, was incubated at 30° C. The evaluation of the reaction products was identical as in the preceding cases. It follows from the results (Table 4) that the cell-free extract of *Brevibacterium ammoniagenes* pronouncedly catalyzes the phosphoribosylation of uracil-2-^{14}C even in the presence of metabolic precursors of 5-phosphoribosyl-1-pyrophosphate. Conversion of uracil-2-^{14}C to uridine-5'-monophosphate-2-^{14}C proceeds, in comparison with the "standard" conditions,

TABLE 3. Time course of deamination of cytosine-2-^{14}C in the presence of the cell-free extract of *Brevibacterium ammoniagenes* [a].

Compound	R_f	Reaction period (h)											
		0	1	2	3	4	5	6	8	10	22	28	48
Start	0.00	0.3	0.3	0.2	0.4	0.6	0.4	0.7	0.9	1.1	6.1	8.2	18.2
Uracil-2-^{14}C	0.63	—	—	—	—	—	—	—	—	—	4.4	9.2	28.0
Cytosine-2-^{14}C	0.77	99.7	99.7	99.8	99.6	99.4	99.6	99.3	99.1	98.9	89.5	82.6	54.3

TABLE 4. Effect of substitution of 5-phosphoribosyl-1-pyrophosphate by its metabolic precursors on the course of synthesis of uridine-5-monophosphate-2-^{14}C catalyzed by the cell-free extract of *Brevibacterium ammoniagenes* [a].

Compound	R_f	Reaction period (h)												
		0	1	2	3	4	5	6	8	10	22	28	46	52
Start	0.00	—	—	—	—	—	—	—	—	—	1.9	8.7	21.3	46.9
Uridine-5′-monophosphate--2-^{14}C	0.32	—	1.8	2.5	4.4	5.1	5.7	6.1	6.9	7.7	12.9	19.1	51.2	53.1
Uracil-2-^{14}C	0.63	100	98.2	97.5	95.6	94.9	93.9	93.1	93.0	92.3	85.2	72.2	27.6	—

[a] Reaction conditions are given in the text.

more slowly, with a maximum of nucleotide production at about 50 % after approximately 50 hours of incubation. In comparison with the "standard" conditions the rising production of the unidentified compound at the chromatogram start may be observed up to a four-fold amount during the final reaction phase, the compound being the only other reaction product besides uridine-5'-monophosphate-2-^{14}C.

A two-fold increase of the concentration of the cell-free extract in the reaction mixture during phosphoribosylation of uracil-2-^{14}C results in remarkable changes in the composition of the reaction products (Table 5). The maximum of uridine 5'-phosphate-2-^{14}C production is only 20 %, formation of the unidentified product at the chromatogram start shows an increasing trend whereas almost 30 % of radioactive uracil-2-^{14}C remains unreacted.

Time course of the enzyme synthesis of orotidine-5'-phosphate in the presence of a cell-free extract of Brevibacterium ammoniagenes.

A mixture of 0.1 ml 2.10^{-1}M Tris-HCl buffer of pH 7.4, 0.1 ml 2.10^{-2}M $MgSO_4$, 0.2 ml 10^{-2}M 5-phosphoribosyl-1-pyrophosphate, 0.1 ml 8.10^{-3}M orotic acid-6-^{14}C, 0.1 ml 2.10^{-3}M 6-azauridine-5'-phosphate and 0.1 ml enzyme preparation, made with distilled water to 1 ml, was incubated at 30° C. The course of the enzyme reaction was evaluated as described above, the results being shown in Table 6. It may be seen that under the reaction conditions an equilibrium is reached within 10 h of incubation when the conversion of orotic acid-6-^{14}C to orotidine-5'-monophosphate-6-^{14}C proceeds by 60 %. During later phases of the reaction one can detect two further reaction products, one of which was identified as uridine-5'-phosphate-6-^{14}C, present in a trace amount. Especially from the point of view of a practical application it can be considered as significant that the inhibition of orotidine-5'-phosphate decarboxylase at the moment of maximum production of orotidylic acid-6-^{14}C is total. The only components of the final reaction mixture at the optimal reaction period are the desired product and the nonreacted substrate.

Discussion.

In connection with studying the production of orotic acid by *Brevibacterium ammoniagenes* CCEB 394 and its mutants the finding was made that unpurified cell-free extracts of the parent strain catalyze with a high efficiency in the presence of 5-phosphoribosyl-1-pyrophosphate the phosphoribosylation of uracil-2-^{14}C. Under optimum conditions of the reaction the effect of degradation enzymes present in the unpurified bacterial extracts is not apparent. Phosphoribosylation of uracil-2-^{14}C then proceeds in such a way that the only product of the final reaction mixture is uridine-5'-phosphate-2-^{14}C. This

TABLE 5. Effect of increasing the enzyme concentration in the reaction mixture on the course of phosphoribosylation of uracil-2-^{14}C by the catalytic effect of the cell-free extract of *Brevibacterium ammoniagenes* using ribose-5-phosphate and adenosine-5′-triphosphate [a].

Compound	Rf	Reaction period (h)												
		0	1	2	3	4	5	6	8	10	22	28	48	52
Start	0.00	0.3	0.7	1.4	1.7	2.1	2.0	2.7	1.8	2.1	2.4	2.5	39.9	55.0
Uridine-5-mono-phosphate-2-^{14}C	0.32	—	—	—	—	—	—	—	2.0	2.2	4.3	4.9	18.6	20.5
Uracil-2-^{14}C	0.63	99.7	99.3	98.6	98.3	97.9	98.0	97.3	96.2	95.7	93.3	92.6	41.5	25.3

[a] Reaction conditions are given in the text.

TABLE 6. Time course of phosphoribosylation of orotic acid-6-^{14}C catalyzed by the cell-free extract of *Brevibacterium ammoniagenes* in the presence of 6-azauridine-5′-phosphate [a].

Compound	R_f	Reaction period (h)										
		0	1	2	4	6	8	10	12	18	28	46
Start	0.00	—	—	—	—	—	—	—	—	2.3	3.6	8.8
Orotidine-5′-monophosphate-6-^{14}C	0.14	—	14.5	23.6	47.2	57.8	58.3	60.8	60.1	59.5	57.4	55.5
Uridine-5′-monophosphate-6-^{14}C	0.32	—	—	—	—	—	—	—	1.1	1.6	1.9	2.6
Orotic acid-6-^{14}C	0.36	100	85.5	76.4	52.8	42.2	41.7	39.2	38.8	36.6	37.1	33.1

[a] Reaction conditions are given in the text.

finding was utilized for developing a simple and efficient method of enzyme synthesis of radiochemically pure uridine-5′-phosphate-2-^{14}C in a quantitative yield [3].

The catalytic effect of unpurified extracts of *Brevibacterium ammoniagenes* on the phosphoribosylation of uracil-2-^{14}C remains intact even in the case that 5-phosphoribosyl-1-pyrophosphate is substituted in the reaction mixture by its metabolic precursors; the synthesis of uridine-5′-phosphate-2-^{14}C proceeds only with a 50 % yield. Even in this case, the spectrum of products of the final reaction mixture is so simple that for an efficient separation of the product a single paper chromatography will do. Increase of the enzyme concentration in the mixture as compared with the optimum is displayed unfavourably by a low production of uridine-5′-monophosphate-2-^{14}C in comparison with the standard reaction conditions.

The catalytic efficiency of the enzyme preparation shows a high specificity. The preparation is inactive for phosphoribosylation of ^{14}C-labelled guanine and cytosine. Phosphoribosylation of adenine-8-^{14}C results under optimal conditions in a 30 % conversion to adenosine-5′-monophosphate-8-^{14}C, the by-products of the final reaction mixture being about 50 % ^{14}C-labelled hypoxanthine, adenosine and nonreacted adenine, hence biologically active and commercially interesting compounds. Under these conditions also the preparation of adenosine-5′-monophosphate-8-^{14}C by the procedure described here has some practical significance.

With all the cases studied the compounds of zero mobility in the system remain unidentified and their increased production usually sets in only during the later phases of enzyme reactions. In view of the important incorporation of the ^{14}C isotope into these compounds during the enzyme syntheses their identification will be taken up subsequently.

The communication is supplemented with a study of phosphoribosylation of orotic acid-6-^{14}C in the same enzyme system but under conditions of complete inhibition of orotidine-5′-phosphate decarboxylase with 6-azauridine-5′-phosphate. At the time optimum of the reaction, orotidine-5′-monophosphate-6-^{14}C is synthetized to 60 % while the only other compound present is the nonreacted orotic acid-6-^{14}C. We are thus dealing with a type of "pure" synthesis where the final reaction mixture contains only the desired product and the nonreacted substrate. This made it possible to isolate orotidine-5′-monophosphate-6-^{14}C in a high yield and in a chromatographically homogeneous form [3].

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