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Preparation of 5-Halogeno Derivatives of Deoxyuridine Labelled in the Base with ^{14}C

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SUMMARY

5-Halogeno derivatives of deoxyuridine labelled in the base with ^{14}C were prepared by a single-step enzymatic synthesis. When using the dicyclohexylammonium salt of 2-deoxy-α-D-ribose-1-phosphate, an enzyme preparation isolated from Escherichia coli B and 5-halogenouracil-2-^{14}C the corresponding deoxyribonucleosides were obtained in a fine yield. The time course of enzymatic reactions and the dependence of yield on concentration of the enzyme preparation and of 2-deoxy-α-D-ribose-1-phosphate were examined. Likewise, the transfer of the deoxyribosyl group between thymidine and 5-halogenouracil was investigated and it was found that with the reactions studied the reaction equillibrium is approximately independent on type of halogen. For isolating the 5-halogeno derivatives of deoxyuridine-2-^{14}C from the reaction mixture we used preparative paper chromatography and obtained compounds of radiochemical purity exceeding 99 %.

INTRODUCTION.

Deoxyribonucleosides labelled with ^{3}H and ^{14}C are new essential for studying the mechanism of biosynthesis of DNA on a quantitative level. For this reason, several methods for the preparation of these compounds have been developed, among them particularly enzymatic procedures attracting much attention by their simplicity and wide applicability.

Synthesis of purine and pyrimidine deoxyribonucleosides by means of transfer of an inactive deoxyribosyl group to a radioactive base, catalyzed by deoxyribosyl transferase (trans-N-deoxyribosylase) is a frequently applied method of preparation of radioactive deoxynucleosides [1-4]. The relatively low specificity of the enzyme makes it possible to use this procedure both for pyrimidine and for purine deoxyribonucleosides. The low degree of con-

version of uracil and thymine to the corresponding deoxyriboside somewhat diminishes the general applicability of the method.

An especially suitable case of enzyme synthesis of thymidine and deoxyuridine is the deoxyribosylation of thymine and uracil in the presence of deoxyribose-1-phosphate catalyzed by pyrimidine deoxyriboside phosphorylase [5-7]. Application of the supernatant fraction of a sonic homogenate of *E-coli* B cells brought up the possibility of synthesizing thymidine-^{14}C and deoxyuridine-^{14}C in this manner [8].

5-Halogeno derivatives of uracil have been studied among other reasons also because of the possibility of replacing thymine in the primary structure of phage and bacterial DNA in connection with the problem of genetic mutations. Experiments with deoxyribosylation of some 5-halogeno derivatives of uracil in the presence of highly purified enzyme of *E-coli* B [9] and of Ehrlich ascites cells [10] gave positive results. In the present communication the previous reports [4-8] are supplemented with a general study of deoxyribosylation of 5-halogeno derivatives of uracil-2-^{14}C in the presence of nonpurified enzyme from *E-coli* B (sonic cell extract). Some kinetic relationships and details of preparation are presented.

EXPERIMENTAL

Chemicals and Radioactive Material.

Nonlabelled bases were products of Lachema (Czechoslovakia). The chromatographic standard of 5-fluorouracil was obtained from Dr. Buděšínský (Research Institute of Pharmacy and Biochemistry). 2-Deoxy-α-D-ribose-l-phosphate as a dicyclohexylammonium salt (further on only deoxyribose-l-phosphate) was a product of Calbiochem (U.S.A.).

The other organic and inorganic chemicals were from Lachema and their purity was of a reagent grade. The starting radioactive compounds were products of the authors, institute and their radiochemical purity was higher than 98 %.

Enzyme.

The enzyme from *Escherichia coli* B was prepared at the Institute of Organic Chemistry and Biochemistry in Prague by Dr. A. Čihák. The preparation procedure was principally identical with a previously published one [23]. Five liter of a synthetic medium containing glucose (composition of the medium is given in ref. [24]), were inoculated with 150 ml of a culture of *E-coli* B, 24 h old. Under intense aeration the culture was propagated for 12-14 h at 37° C. Growth was terminated by cooling to a low temperature. The cells were then centrifuged, washed with 0,9 % sodium chloride, cooled to 3° C and suspended in 10 ml of the same solution. The suspension was frozen and cells were dis-

rupted at -40° C in a bacterial press. The crushed cells were suspended in 50 ml 0. 1M Tris buffer of pH 7.4. The cell debris was removed by centrifugation at 0° C. The opalescent supernatant was frozen and kept at -15° C.

The supernatant was not further purified and was used directly for enzyme reactions. Study of the Kinetics of Enzymatic Deoxyribosidation of 5-Fluorouracil-2-^{14}C with a Preparation from *E-coli* B.

(a) General procedure.

Deoxyribosidation of 5-halogenouracil-2-^{14}C took place in a medium of 0.1M Tris buffer at pH 7.4. The reaction temperature during all the experiments was 37° C. The experiments were protected from bacterial infection with a layer of toluene. The enzyme reactions were terminated by diluting the sample with ethanol. At definite time intervals aliquots were removed from each sample and analyzed by paper chromatography. The time dependence of the course of enzyme reaction on the amount of enzyme and of deoxyribose-1-phosphate were examined.

(b) Analysis of the Reaction Mixture.

Samples of reaction mixtures were analyzed by paper chromatography on Whatman no. 3 in several chromatographic systems together with authentic standards (Table 1). The standards were detected in UV light (Chromatolite), radioactive compounds were detected with Agfa-Texo-X-ray film. The radioactivity of compounds on the chromatogram was counted in a 2 π counter.

TABLE 1. Paper Chromatography of 5-Halogeno Derivatives of Deoxyuridine-2-^{14}C

Compound	R_F Value in Solvent System[a]		
	A	B	C
5-Iodouracil-2-^{14}C	0,45	0,55	0,43
5-Iododeoxyuridine-2-^{14}C	0,31	0,43	0,30
5-Bromouracil-2-^{14}C	0,34	0,34	0,33
5-Bromodeoxyuridine-2-^{14}C	0,24	0,28	0,22
5-Fluorouracil-2-^{14}C	0,41	0,50	0,41
5-Fluorodeoxyuridine-2-^{14}C	0,30	0,38	0,28

[a] A : ethyl acetate saturated with water; B : 1-butanol-1-propanol-ammonia-water (7 : 5 : 7 : 2); C : ethyl acetate-acetic acid-water (4 : 1 : 5).

(c) Examples of Determining Kinetic Relationships.

I. Deoxyribosidation of 5-fluorouracil-2-^{14}C at a constant molar ratio of deoxyribose-1-phosphate and 5-fluorouracil-2-^{14}C (3 : 1) with rising concentrations of the enzyme.

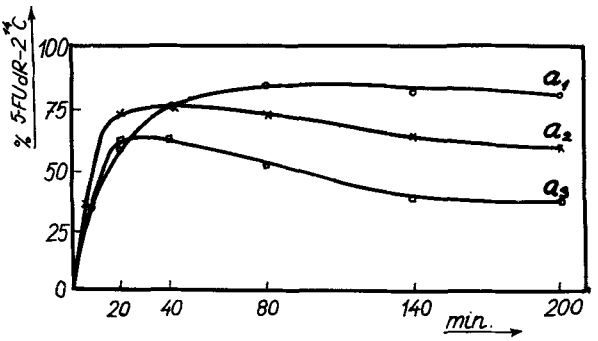

FIG. 1.

25.0 μCi 5-fluorouracil-2-^{14}C (0.130 mg, 1.00 μmol) of specific activity 25.0 mCi/mmole were incubated with 1.1 ml 0.1M Tris buffer and 1.2 mg (2.92 μmole) deoxyribose-1-phosphate and (a_1) 0.025 ml, (a_2) 0.08 ml, (a_3) 0.20 ml enzyme preparation for 200 min. Samples were withdrawn during the incubation. The time dependence of the yield of 5-fluoro-2′,-deoxyuridine-2-^{14}C for the individual reactions in Fig. 1.

II. Deoxyribosidation of 5-bromouracil-2-^{14}C at a constant molar ratio of deoxyribose-1-phosphate and 5-bromouracil-2-^{14}C (3 : 1) and increasing concentrations of the enzyme.

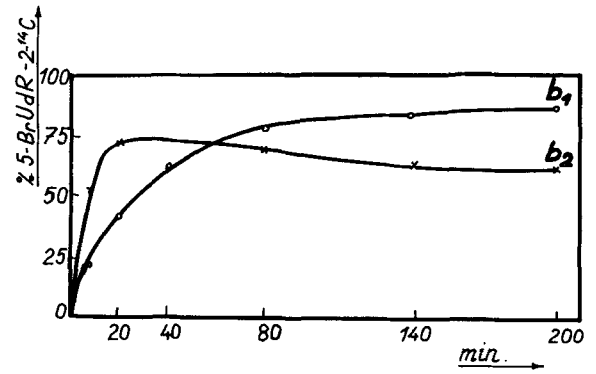

FIG. 2.

43.O μCi 5-bromouracil-2-^{14}C (0.192 mg; 1.00 μmole) of specific activity 43.0 mCi/μmole were incubated in 1.0 ml 0.1M Tris buffer with 1.2 mg (2.92 μmole) deoxyribose-1-phosphate and (b_1) 0.08 ml, (b_2) 0.2 ml enzyme preparation. During the reaction which took place for 200 min, samples were withdrawn and analyzed.

The time dependence of the yield 5-bromo-2-deoxyuridine-2-^{14}C for the individual reactions is shown in Fig. 2.

III. Deoxyribosidation of 5-iodouracil-2-^{14}C at a constant molar ratio of deoxyribose-1-phosphate and 5-iodouracil-2-^{14}C (3 : 1) and increasing concentrations of the enzyme.

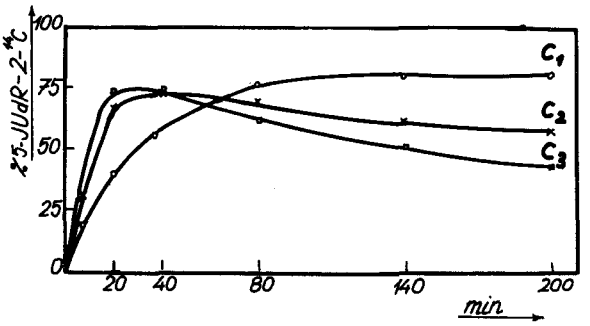

FIG. 3.

45.0 μCi 5-iodouracil-2-^{14}C (0.238 mg; 1.00 μmole) of specific activity 45.0 mCi/mmole were incubated in 1.0 ml 0.1M Tris buffer with 1.2 mg (2.92 μmole) deoxyribose-1-phosphate and (c_1) 0.025 ml, (c_2) 0.08 ml, (c_3) 0.20 ml enzyme preparation for 200 min. Samples were withdrawn during the reaction and analyzed. The time dependence of the yield of 5-iodo-2′-deoxyuridine-2-^{14}C for the individual reactions is shown in Fig. 3.

IV. Dependence of deoxyribosidation of 5-fluorouracil-2-^{14}C on the concentration of deoxyribose-1-phosphate at a constant enzyme concentration.

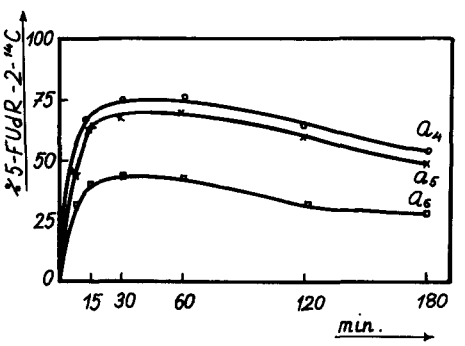

FIG. 4.

25.0 μCi 5-fluorouracil-2-^{14}C (0.130 mg; 1.00 μmole) of specific activity 25.0 mCi/mmole were incubated with 0.9 ml 0.1 Tris buffer and (a_4) 1.2 mg (2.92 μmole), (a_5) 0.8 mg (1.94 μmole) and (a_6) 0.4 mg (0.97 μmole) deoxyribose-1-phosphate in the presence of 0.1 ml enzyme preparation. The reaction took place for 180 min. Samples were withdrawn in the course of the reaction and analyzed. The time dependence of the yield of 5-fluoro-2′-deoxyuridine-2-^{14}C on the concentration of deoxyribose-1-phosphate is shown in Fig. 4.

V. Dependence of deoxyribosidation of 5-bromouracil-2-^{14}C on the concentration of deoxyribose-1-phosphate at a constant enzyme concentration.

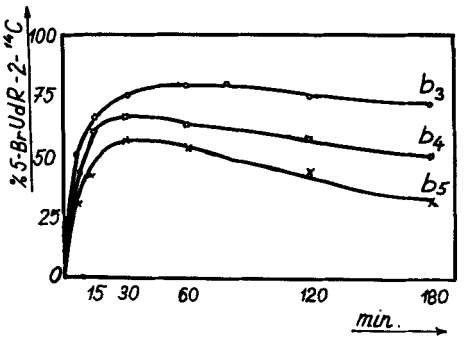

FIG. 5.

43.0 μCi 5-bromouracil-2-^{14}C (0.192 mg; 1.00 μmole) of specific activity 43.0 mCi/mmole were incubated with 0.8 ml 0.1M Tris buffer and (b_3) 1.2 mg (2.92 mmole), (b_4) 0.8 mg (1.94 μmole), (b_5) 0.4 mg (0.97 μmole) deoxyribose-1-phosphate in the presence of 0.1 ml enzyme preparation. The enzyme reaction took place for 180 min. Samples were withdrawn during the reaction and analyzed. The time dependence of the yield of 5-bromo-2′-deoxyuridine-2-^{14}C for reactions conditions (b_3), (b_4) and (b_5) is shown in Fig. 5.

VI. Dependence of the deoxyribosidation of 5-iodouracil-2-^{14}C on the concentration of deoxyribose-1-phosphate at a constant concentration of the enzyme.

45.0 μCi 5-iodouracil-2-^{14}C (0.238 mg; 1.00 μmole) were incubated with 1.0 ml 0.1M Tris buffer and (c_4) 1.2 mg (2.92 μmole), (c_5) 0.8 mg (1.94 μmole (c_6) 0.4 mg (0.97 μmole) deoxyribose-1-phosphate in the presence of 0.1 ml enzyme preparation. The reaction took place for 180 min. Samples were withdrawn at time intervals and analyzed. The time dependence of the yield of 5-iodo-2′-deoxyuridine-2-^{14}C is shown in Fig. 6.

VII. Time dependence of transdeoxyribosidation in a system of 5-halogenouracil-2-^{14}C-thymidine catalyzed by an enzyme preparation from *E-coli* B.

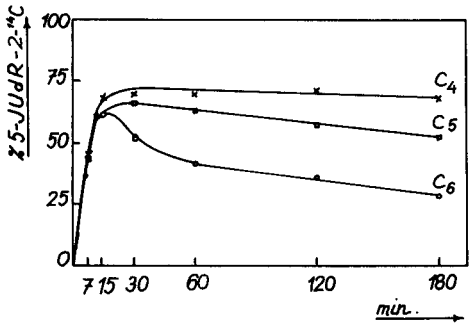

FIG. 6.

1.0 μmole 5-halogenouracil-2-^{14}C of specific activity shown in Table 2 and 1.0 μmole thymidine dissolved in 0.7 ml 0.1M Tris buffer were placed in an ampoule with 0.2 ml enzyme preparation. The ampoules were heated to 37° C in a thermostat. Samples were withdrawn at time intervals and analyzed The time dependence of transdeoxyribosidation for the individual experiments is shown in Table 2.

TABLE 2

Reaction period	Yields[a] in %		
	5-FdUR	5-BrdUR	5-IdUR
8	5.7	7.1	8.1
20	14.2	15.3	16.7
38	15.2	16.1	17.2
78	16.6	16.0	18.1
138	16.1	15.19	17.3
200	15.5	15.4	15.0

[a] The activity of 5-halogeno-2′-deoxyuridine-2-^{14}C is expressed in per cent of activity of the reaction mixture.

Specific activity : 5-fluorouracil-2-^{14}C 25.0 mCi/mmole, 5-bromouracil-2-^{14}C 43.0 mCi/mmole, 5-iodouracil-2-^{14}C 45.0 mCi/mmole.

DISCUSSION.

The presence of pyrimidine phosphorylase in *E-coli* was reported by Manson and Lampen [11]. It was isolated in a pure state by Razzel and Khorana [9] who demonstrated that after a careful purification it catalyzes specifically the

transfer of deoxyribose-1-phosphate to pyrimidine bases. We studied previously the possibility of using the sonic extract from *E-coli* B for the preparation of thymidine-2-^{14}C and uridine-2-^{14}C. It was found now that the same enzyme preparation can be used for preparing 5-halogenoderivatives of 2′-deoxyuridine-2-^{14}C. We attempted to accumulate kinetic data that must be known for attaining satisfactory yields. We followed the effect of concentration of deoxyribose-1-phosphate on the yield of 5-halogeno-2′-deoxyribonucleosides and the effect of the amount of enzyme. One can conclude that the concentration of deoxyribose-1-phosphate has a significant effect and that to attain fine yields (over 70 %) of 5-halogeno-2′-deoxyuridine-2-^{14}C one must use a several-fold higher molar concentration as compared with 5-halogenouracil-2-^{14}C. In all cases we obtained very rapidly the optimal yield which decreased slightly with time (see Figs. 1-3), the drop not being significantly dependent on the concentration of deoxyribose-1-phosphate. During deoxyribosidation of 5-halogenouracil-2-^{14}C at the conditions used here there is no pronounced effect of the halogen in position 5 and the kinetic relationships for the fluoro, bromo and iodo derivatives have the same character as was found previously when studying the effect of enzyme concentration on deoxyribosidation of uracil-2-^{14}C and thymine-2-^{14}C. When following the effect of concentration of the enzyme preparation it was found that the enzyme concentration has a pronounced effect on the reaction rate. At lower concentrations, the reaction rate was substantially lower but the yield of 5-halogeno-2′-deoxyuridine-2-^{14}C was higher in all cases.

We studied also the ability of the enzyme preparation to catalyse the transfer of the deoxyribosyl group from thymidine to 5-halogenouracil-2-^{14}C (table 2). It was found that the trans-N-deoxyribosylase activity contained in a sonic extract of *E-coli* B makes it also possible to transfer the deoxyribosyl group from thymidine to 5-halogenouracil-2-^{14}C, the effect of the halogen playing no great role.

The kinetic relationships studied here show that for preparing radioactively labelled 5-halogeno derivatives of 2′-deoxyuridine-2-^{14}C one can use with advantage deoxyribose-1-phosphate and a sonic extract of *E. coli* B. By determining the enzyme a activity one can also determine the optimum reaction conditions and prepare 5-halogeno-2′-uridine labelled with the radioisotope in a fine yield and at a high radiochemical purity.

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