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Preparation of Thymidine and Deoxyuridine Labelled with ^{14}C in Base

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SUMMARY

Deoxyribosides of thymine and uracil labelled with ^{14}C of specific activity identical with that of the original radioactive base were prepared by single-step enzymatic synthesis. When employing 2-deoxy-α-D-riboso-l-phosphate of dicyclohexylamine salt, the enzymatic preparation isolated from Escherichia coli B *gives satisfactory yields in the synthesis of both deoxyribonucleosides. Studies were made of the time course of reactions, as well as of the effects of some components on the yields. It has been found that the enzymatic preparation from* Escherichia coli B *facilitates the transfer of the deoxyribosylase group between thymine and thymidine. The employed technique separation allows preparation of radioactive uracil and thymine deoxyribosides of a radiochemical purity exceeding 99 per cent.*

INTRODUCTION.

For microscale preparation of deoxyribonucleosides it is convenient to employ enzymatic synthesis. So far, studies were made of the catalytic effect of deoxyribosyl transferase (trans-N-deoxyribosylase) isolated from bacterial sources [1-10] and mammal tissues [11-13]. The substrate specificity of this enzyme is very low so that the enzyme catalyzes the transfer of deoxyribosyl group into the purine and pyrimidine bases according to the following scheme :

$$\text{base}_1 + \text{base}_2\text{—deoxyriboside} \underset{\text{enzyme}}{\rightleftharpoons} \text{base}_1\text{—deoxyriboside} + \text{base}_2$$

Another type of catalyzing enzyme which can be employed for the synthesis of deoxyribosides, is the nucleoside phosphorylase which is isolated

from mammal tissue, e.g. rats [14-16], horse [17] and beef [18] liver, as well as from microorganismus, e.g. the cells of *Escherichia coli B* [19, 20]. On the contrary, this enzyme exhibits a considerable substrate specificity, as it catalyzes primarily the transfer of deoxyribosylasic group onto pyrimidine bases according to the reaction

$$\text{base} + \text{deoxyriboso-1-phosphate} \underset{\text{enzyme}}{\rightleftharpoons} \text{deoxyribonucleoside} + \text{orthophosphate}$$

as well as the transfer of deoxysibosylase group of pyrimidine deoxyribonucleoside into the pyrimidine base in exchange reactions of the following types :

$$T^X + TdR \rightleftharpoons T^X dR + T \qquad (21)$$

$$\text{or} \quad U^X + UdR \rightleftharpoons U^X dR + U \qquad (22)$$

Deoxyribonucleosides labelled with a suitable isotope play an important part in the study of DNA metabolism in organismus. A great deal of attention is therefore paid to the preparation of ^{14}C-labelled deoxyribonucleosides by the above mentioned enzymatic reactions [7-11, 17, 21, 22].

Both types of the above enzymatic reactions are reversible ones. It is therefore of utmost importance to know the kinetic relationships of these reactions in order to be able to affect the yields of radioactive products. In the case of trans-N-deoxyribosylase isolated from the microorganism *Lactobacillus*, the most common enzyme source, these data are available [40, 11].

In this study the authors have tried to collect similar data on the relations for the nucleoside phosphorylase, isolated from *Escherichia coli* cells, as the most easily available source of this enzyme in laboratory work.

EXPERIMENTAL.

Chemicals and radioactive materials.

The non-active bases of nucleic acids were supplied by the firm Lachema (Czechoslovakia). Uracil and thymine deoxyribonucleosides, as well as 2-deoxy-α-D-riboso-1-phosphate dicyclohexylamine salt, are commercial products of the firm Calbiochem (USA). The reagents are designated as chromatographically homogeneous. All other organic and inorganic chemicals were supplied by Lachema in the A.R. quality. The initial radioactive substrates for enzymatic reactions were uracil-2-^{14}C and thymine-2-^{14}C. Both radioactive bases are products of the firm UVVVR (Czechoslovakia) and their radiochemical purity is guaranteed as exceeding 98 per cent.

Enzyme.

The sonic extract containing nucleoside phosphorylase activity was prepared from *Escherichia coli B* cells. The preparation procedure was principally identical with that already described elsewhere. Five litres of synthetic

medium containing glucose (for medium composition refer to 24) were inoculated with 150 ml of *E. coli B* culture, 24 hours old. With intensive aeration the culture was cultivated for 14 hr at 37° C. The growth was concluded by cooling to low temperature. The cells were then centrifuged and washed with 0.9 per cent sodium chloride solution cooled to 3° C. The washed cells were suspended in 50 ml of 0.1 M Tris buffer solution having pH 7.4, and broken up with ultrasound in a MSE apparatus using the maximum output (input 60 Watt, frequency 20×10^3 c/s) for 5 minutes at 0° C. The cell debris was centrifuged, and the opalescent supernatant (45 ml) was frozen and stored at —15° C.

The supernatant was not further purified and was employed in this form for enzymatic reactions.

Study of the kinetics of enzymatic deoxyribosidation of ^{14}C labelled uracil and thymine by means of Escherichia coli B *enzyme.*

a) General procedure.

Deoxyribosidation of uracil-2-^{14}C (or thymine-2-^{14}C) by means of deoxyriboso-1-phosphate and the *E. coli* cell enzyme was carried out in Tris buffer at pH 7.4. In addition to the time studies of the reaction course the authors have also determined the optimum conditions for an enzymatic reaction from the standpoint of the amount of enzyme present, and the mutual molar ratio of deoxyriboso-1-phosphate and the radioactive base. All the enzymatic reactions took place at 37° C. The solutions were kept in sealed vials and protected from bacterial contamination by a toluene layer. The enzymatic reactions were concluded by a short heating of the solutions in a boiling water bath. At certain time intervals samples were taken from the incubated solutions; the above mentioned relationships were determined by paper chromatographic analysis of these samples.

b) Analysis of reaction mixtures.

Samples of reaction mixtures were analyzed by paper chromatography on Whatman No. 3 paper in several solvent systems simultaneously with the corresponding standards of the initial substances and those of the reaction products (see Table 1). The standards of nucleic acid components were detected as extinguishing spots in UV light of a Chromatolite lamp. The radioactive substances were detected by means of Agfa-Texo X-ray films. The intensity of radioactivity of substances on the chromatogram was determined by their measuring in a 2π counter.

All analyses were carried out on Whatman 3 paper, length 47 cm, temperature 20 to 22° C.

TABLE 1. Paper chromatography of uracil, deoxyuridine, thymine and thymidine. R_F values [a]

Components	Solvent system [b]		
	A	B	C
Uracil	0.29	0.39	0.31
Deoxyuridine	0.14	0.41	—
Thymine	0.55	0.55	0.46
Thymidine	0.27	0.54	0.30

[a] The R_F values of the bases and desoxyribosides have been obtained by multiple developing in ethylacetate saturated with water, the total time of developing being 18 hours. The authors lay stress upon the fact that developing in this solvent system is sensitive to the humidity of the paper, the saturation of the chamber with solvent vapor and temperature changes during development of the chromatogram. In the system n-butanol saturated with water, our R_F values differ from the published values by 0.03 and 0.06 resp. The values reported are average from a series of experiments.

[b] A : Water-saturated ethyl acetate
B : *n*-Butyl alcohol saturated with water
C : Ethyl acetate — acetic acid — water (4 : 1 : 5).

c) Examples of kinetics relationship determination.

I. Deoxyribosidation of uracil-2-^{14}C at a constant molar ratio of deoxyriboso-1-phosphate and uracil-2-^{14}C (3 : 1) and an increasing enzyme concentration.

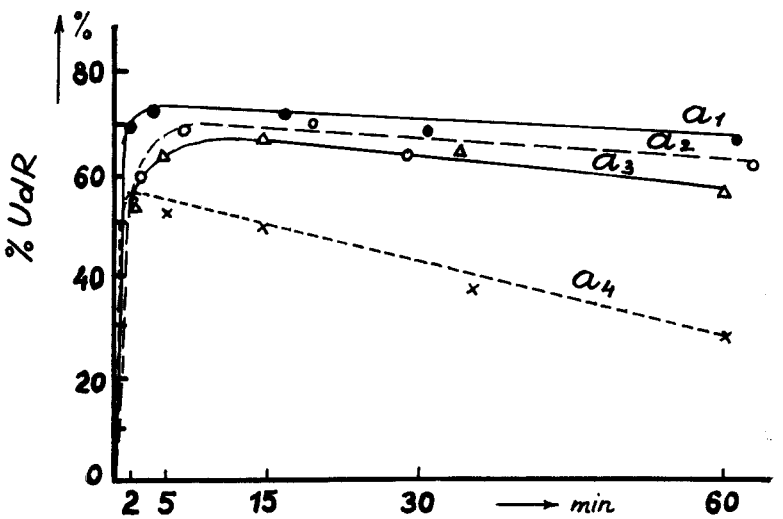

FIG. 1.

29.0 μCi of uracil-2-^{14}C (0.112 mg; 1.00 μMol) with specific activity of 29.0 mCi/mMol was incubated in 0.7 ml of 0.2 M Tris buffer with 1.20 mg (2.92 μMol) of deoxyriboso-1-phosphate and a_1) 0.025 ml; a_2) 0.05 ml; a_3) 0.10 ml; a_4) 0.20 ml of enzyme at 37° C for 60 minutes. Samples were taken and analysed in the course of the reaction. The relationship between the yield of deoxyuridine-2-^{14}C at increasing amounts of enzyme and time for the individual reactions is shown in Figure 1.

II. Deoxyribosidation of uracil-2-^{14}C at constant enzyme concentration and increasing ratio of deoxyriboso-1-phosphate to uracil-2-^{14}C.

29.0 μCi of uracil-2-^{14}C (0.112 mg; 1,000 μM) specific activity 29.0 mCi/mMol in 1 ml of 0.2 M Tris buffer was incubated with 0.05 ml of enzyme and b_1) 0.40 mg (0.972 μMol), b_2) 0.80 mg (1,944 μMol) and b_3) 1,20 mg (2.916 μMol) of deoxyriboso-1-phosphate. The incubation lasted 1 hour. Samples were taken and analysed in the course of the reaction. The relationship between the deoxyuridine-2-^{14}C yield and time at increasing deoxyriboso-1-phosphate is shown in Figure 2.

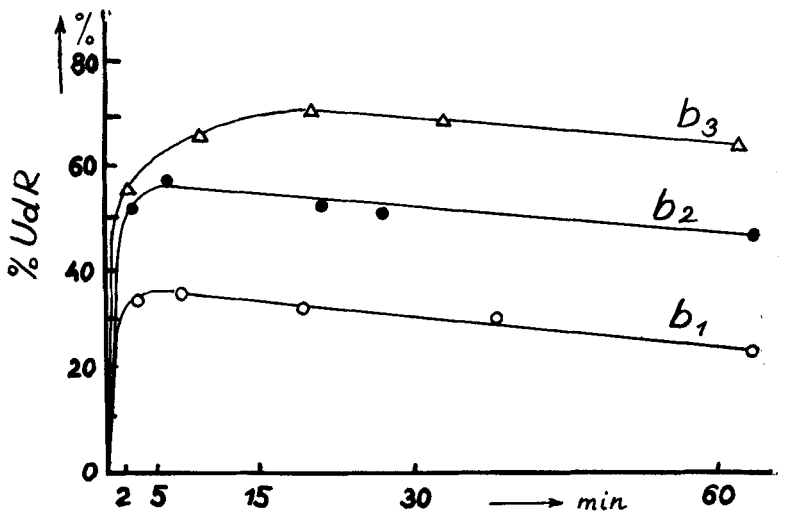

FIG. 2.

III. Deoxyribosidation of thymine-2-^{14}C at constant molar ratio of deoxyriboso-1-phosphate and thymine-2-^{14}C and an increasing enzyme concentration.

To 10 μCi of thymine-2-^{14}C (0.065 mg; 0.515 μMol) with a specific activity of 19.4 μCi/μMol in 0.1 ml of distilled water 0.2 ml of 0.1 M Tris buffer, 0.1 ml of 0.0196 M $MgCl_2$ solution and 0.400 mg (0.969 μMol) of deoxyriboso-phosphate were added; the mixture was incubated with the enzyme for 30 minutes at 37° C. The amount of enzyme employed and the thymidine-2-^{14}C yields are plotted in Table 2.

TABLE 2. Relationship of thymidine-2-^{14}C yield and the amount of enzyme (for reaction conditions III).

Amount of enzyme	per cent of T*d*R-2-^{14}C yielded
0.01	51.0
0.025	54.1
0.05	49.8
0.15	20.2
0.2	17.2
0.3	12.5
0.5	9.6

IV. Time relationship of deoxyribosidation in the thymine-2-$^{14}C \rightleftharpoons$ thymidine system under the effects of enzyme isolated from *Escherichia coli B.*

To 30 μCi of thymine-2-^{14}C (0.200 mg; 1.586 μMol) with a specific activity of 18.91 mCi/mMol dissolved in 0.2 ml of distilled water, 0.4 ml of 0.1 M Tris buffer, 0.2 ml of 0.0196 N barium chloride solution, 0.384 mg (1.587 μMol) of thymidine in 0.2 ml of distilled water and 0.8 ml of phosphate buffer were added; the mixture was incubated with 0.1 ml of the enzyme for 180 minutes at 37° C. Samples in which the content of thymidine-2-^{14}C was determined, were taken from the reaction mixture at certain time intervals.

The results are given in Table 3.

TABLE 3. Time relationship of deoxyribosidation in the system thymine-2-^{14}C thymidine under the effect of enzyme isolated from *Escherichia coli B.*

Cultivation time (min)	T*d*R-2-^{14}C yield (per cent)
15	28.4
30	30.6
60	34.8
120	37.2
180	32.5

V. Deoxyribosidation of thymine-2-^{14}C at constant enzyme concentration (0.01 ml) and an increasing molar ratio of deoxyriboso-1-phosphate/thymine-2-^{14}C.

10.3 μCi of thymine-2-^{14}C (0.050 mg; 0.396 μMol) with a specific activity of 26.1 mCi/mMol in 0.1 ml of distilled water was added to 0.2 ml of 0.1 M Tris buffer solution, 0.1 ml of 0.196 M magnesium chloride, 0.01 ml of enzyme,

TABLE 4. Relationship between the yield of thymidine-2-^{14}C and the molar ratio deoxyriboso-1-phosphate/thymine-2-^{14}C.

Deoxyriboso-1-phosphate / thymine-2-^{14}C	Thymidine-2-^{14}C yield (per cent)
2.44	70.6
4.88	76.9
7.32	77.4

and the mixture was incubated with deoxyriboso-1-phosphate (the amount is given in Table 4) for 30 minutes at 37° C. The amount of thymidine-2-^{14}C was determined in the reaction mixture.

The results are given in Table 4.

VI. Determination of the time course of thymine-2-^{14}C deoxyribosidation.

To 40.1 μCi thymine-2-^{14}C (0.200 mg; 1.584 μMol) of a specific activity of 26.1 mCi/mMol in 0.4 ml of distilled water 0.8 ml of 0.1 M Tris buffer, 0.4 ml of 0.0196 M magnesium chloride solution and 1.600 mg (3.8796 μMol) deoxyriboso-1-phosphate in 0.4 ml of distilled water was added and the mixture was incubated with 0.04 ml of enzyme for 24 hours at 37° C. The time intervals of samples taken from the reaction mixture and the results of analyses of the reaction mixtures are given in Table 5.

TABLE 5. Time relationship of thymine-2-^{14}C deoxyribosidation [a]

	Reaction time (hrs)						
	0.25	0.5	1	2	4	8	24
Start	3.8	4.4	4.4	3.2	3.2	10.7	—
Thymidine-2-^{14}C	78.7	69.2	70.5	73.4	67.7	28.5	—
Thymine-2-^{14}C	17.5	26.4	25.1	23.4	29.1	60.9	100

[a] Radioactivity of the individual components is expressed in per cent of the reaction mixture radioactivity on the chromatogram.

DISCUSSION.

Although the preparation of highly purified nucleoside phosphorylase from *Escherichia coli B* was already described, it has been proved that a mere sonic extract from *E. coli B* cells contains the nucleoside phosphorylase of

satisfactory enzymatic activity, and that it can be used for deoxyribosidation in common laboratory practice. The results shown in the experimental section prove that to attain a good yield of deoxyuridine and thymidine labelled with ^{14}C (or tritium) it is necessary to find the optimum ratio of the radioactive base, deoxyriboso-1-phosphate, as well as of the enzyme preparation employed. Above all it is obvious that the effect of deoxyriboso-1-phosphate concentration (in terms of that of the base) on the course of the reaction is extraordinarily great and satisfactory yields of deoxyriboside require excessive amounts of the deoxyribose donor. A similar relationship has also been found in the case of trans-N-deoxyribosylase [10]. However, a great excess of deoxyriboso-1-phosphate has no considerable effect on the yield of deoxyriboside. On the other hand the effect of enzyme is marked especially at low concentrations. The deoxyribonucleoside yield decreases when larger amounts of the enzymatic preparation are employed.

The studies of thymine-2-^{14}C deoxyribosidation in terms of time have shown that the course of enzymatic synthesis is very rapid and that the reversible reaction begins to prevail after long reaction periods. After 24 hours, thymine-2-^{14}C only was isolated from the reaction mixture again. This can be caused by a small stability of deoxyriboso-1-phosphate under the reaction conditions.

The relationship between deoxyribosidation and the amount of enzyme at constant thymine-2-^{14}C : deoxyriboso-1-phosphate ratio indicates that the yield is very satisfactory at low enzyme concentrations in case of excess of deoxyriboso-1-phosphate. An eightfold increase in the optimum enzyme concentration results in a reduction of thymine-2-^{14}C yield to about one third of the original value. For this reason it is necessary to determine and test the enzyme activity. It has already been found that the sonic extract from *E. coli B* also contains trans-N-deoxyribosylase activity in addition to the nucleoside phosphorylase. Studies of trans-N-deoxyribosylase activity of the enzymatic preparation made by the authors have shown that when using thymidine as a deoxyribose donor, the transfer proceeds very rapidly and the system approaches a state of equilibrium. When using deoxyadenosine as a deoxyribose donor, the yields of thymidine-2-^{14}C are considerably lower which proves that trans-N-deoxyribosylase activity present in an extract of *E. coli B* primarily catalyzes the transfer between the pyrimidine derivatives.

Experimental evaluation of thymine-2-^{14}C deoxyribosidation has shown that enzymatic reactions of this type can be carried out within a wide range from μMol up to the order of 100 μMol with quite satisfactory yields.

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REFERENCES

1. MAC NUTT, W. S. — *Biochem. J.*, **50** : 384 (1952).
2. HOFFMAN, C. E. — *Federation Proc.*, **11** : 231 (1952).
3. ROUSCH, A. H. and BETZ, R. F. — *J. Biol. Chem.*, **233** : 261 (1958).
4. KANDA, M. and TAKAGI, Y. — *J. Biochem. (Japan)*, **46** : 725 (1959).
5. MINGHETTI, A. — *Ital. J. Biochem.*, **8** : 224 (1960).
6. WILLIAMS, D. L. — *Canad. J. Chem.*, **40** : 1742 (1962).
7. BEEK, W. S. and LEVIN, M. — *J. Biol. Chem.*, **238** : 702 (1963).
8. KÁRA, J. and ŠORM, F. — *Coll. Czech. Chem. Commun.*, **28** : 1441 (1963).
9. NEJEDLÝ, Z., FILIP, J. and EKL, J. — *J. Labelled Compounds* (in press).
10. CARDINAUD, R. and VISWANATHAN, K. V. — *J. Labelled Compounds*, **2** : 35 (1966).
11. DE VERDIER, C. H. and VAN POTTER, R. — *J. Nat. Cancer Inst.*, **24** : 13 (1960).
12. ZIMMERMAN, M. and SEIDENBERG, J. — *J. Biol. Chem.*, **239** : 2618 (1964).
13. ZIMMERMAN, M. — *J. Biol. Chem.*, **239** : 2622 (1964).
14. FRIEDKIN, M. and KALCKAR, H. M. — *J. Biol. Chem.*, **184** : 437 (1950).
15. FRIEDKIN, M. — *J. Biol. Chem.*, **184** : 449 (1950).
16. HOFF-JÖRGENSEN, E., FRIEDKIN, M. and KALCKAR, H. M. — *J. Biol. Chem.*, **184** : 461 (1950).
17. FRIEDKIN, M. and DE ROBERTS, W. — *J. Biol. Chem.*, **207** : 257 (1954)
18. BIEBER, A. L. and SARTORELLI, A. C. — *Nature*, **201** : 624 (1964).
19. MANSON, L. A. and LAMPEN, J. O. — *J. Biol. Chem.*, **193** : 539 (1951).
20. RAZZEL, W. E. and KHORANA, H. G. — *Biochim. Biophys. Acta*, **28** : 562 (1958).
21. SEKIGUCHI, T. and YOSHIKAWA, H. — *J. Biochem.*, **46** : 11, 1505 (1959).
22. SKÖLD, O. — *Biochim. Biophys. Acta*, **44** : 1 (1960).
23. ČIHÁK, A., ŠKODA, J. and ŠORM, F. — *Coll. Czech. Chem. Commun.*, **29** : 814 (1964).
24. ŠKODA, J., HESS, V. F. and ŠORM, F. — *Coll. Czech. Chem. Commun.*, **22** : 1130 (1957)