SYNTHESIS OF CARBON-14 LABELED DEOXYRIBONUCLEOSIDES[1]

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DISCLAIMER

ABSTRACT

The enzymatic transfer of deoxyribose from a purine or a pyrimidine deoxyriboside to a carbon-14 labeled purine or pyrimidine base affords an efficient means of preparing labeled deoxyribonucleosides. Deoxyadenosine-8-C^{14}, deoxyguanosine-8-C^{14}, deoxycytidine-2-C^{14}, and thymidine-2-C^{14}, with the natural ß-configuration, were prepared in good yields and isolated in radiochemically pure form by large-scale paper chromatography.

INTRODUCTION

Carbon-14 labeled deoxyribonucleosides are not, as yet, commercially available. Strictly chemical syntheses of 2'-deoxyribosides (a) by the reduction at C2' of ribofuranosyl-purines (1) and -pyrimidines (2,3); (b) through condensation of acylated 2-deoxyribosyl halides with suitably substituted purines (4-6) and pyrimidines (7); or (c) by the direct condensation of 2-deoxyribose with purines and pyrimidines catalyzed by polyphosphoric acid ester (8) are, in general, attended by requirements for difficultly obtainable intermediates, low yields, or the formation of both the α- and ß-anomers. The ß-anomer is the desired natural product.

In terms of high yields of solely the desired ß-anomer and probable time consumption, enzymatic methods of synthesis appear much more of interest than the above chemical methods. The phosphorylase enzymes (9-13) give excellent yields of deoxyribosides from deoxyribose-1-phosphate and certain purines and pyrimidines, but the enzyme from a given tissue and species of organism is too specific and a number of enzyme preparations would be required to obtain the desired products. The trans-N-glycosidase first described by MacNutt (14), which was more specifically named trans-N-deoxyribosylase and further characterized by Roush and Betz (15), appeared to be the enzyme of choice, since it is nonspecific and transfers 2-deoxyribose

from any deoxyriboside to a new purine or pyrimidine base, according to the following equilibrium:

$$\text{base}_1 + \text{base}_2 \text{ deoxyriboside} \rightleftharpoons \text{base}_2 + \text{base}_1 \text{ deoxyriboside}$$

This communication describes the enzymatic synthesis and isolation of 2'-deoxyadenosine-8-C^{14} (I), 2'-deoxyguanosine-8-C^{14} (II), 2'-deoxycytosine-2-C^{14} (III), and thymidine-2-C^{14} (IV).

EXPERIMENTAL

Carbon-14 Assays

Radiochemical yields were determined, with a Tri-Carb spectrometer,* by liquid scintillation counting of suitable aliquots in 15 ml of a dioxane system [naphthalene, 125 g/l.; 2,5-diphenyloxazole (PPO), 7.5 g/l.; and 2,2'-p-phenylenebis-(5-phenyloxazole) (POPOP), 0.375 g/l.] (16).

Paper Chromatography

Reaction mixtures were separated by chromatography on 18 x 44-in. sheets of Whatman No. 3 paper, and the chromatograms were developed with Hems' (17) solvent (85% saturated ammonium bicarbonate) in a combined descending and ascending manner. The paper was attached at

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*Model 314EX, Packard Instrument Company, Inc., LaGrange, Illinois.

both ends to supports in a Chromatocab. This procedure gave good separation of all components, including the pairs: thymine-thymidine and cytosine-deoxycytidine. Upon rechromatography of aliquots of the isolated products on 1-1/2 x 44-in. strips of Whatman No. 3 MM paper, only one ultraviolet-absorbing spot was observed, in each case. The only radioactivity which was observed with a windowless gas-flow strip scanning device* was concentrated in these spots.

R_f values for the compounds encountered in this work are shown in Table I.

Enzymes

Trans-N-deoxyribosylase enzyme was extracted from sonically disrupted Lactobacillus helveticus (ATCC 12046) bacteria with 0.1 M phosphate buffer (pH 6) and partially purified, through the first ammonium sulfate precipitation step, as described by Roush and Betz (15). The protein which precipitated between 35% and 70% of saturation was redissolved in distilled water and dialyzed against distilled water at 4°C until free of sulfate ion. The final solution contained 6 mg of protein per ml, as determined by the method of Lowry et al. (18).

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*Model D-47, Chicago-Nuclear Corporation, Chicago, Illinois.

An auxiliary enzyme, xanthine oxidase, was prepared from fresh, whole cream according to the procedure of Horecker and Heppel (19). Oxidase activity was determined spectrophotometrically with hypoxanthine as substrate. The uric acid produced absorbs strongly at 293 mμ, and there is no interference from purines or pyrimidines or their deoxyribosides at this wavelength.

The specific activity of the transferase enzyme was also determined spectrophotometrically by a modification of the auxiliary enzyme procedure of Kalckar (20). In the transfer reaction between deoxyadenosine and hypoxanthine to form deoxyinosine, the rate of disappearance of hypoxanthine was measured with xanthine oxidase. At a hypoxanthine concentration of 101.3 μg/ml and a molar ratio of deoxyriboside to free purine base of 5 to 1 in pH 6.1 phosphate buffer at 37°C, the initial rate of disappearance of hypoxanthine was 13.13 μmoles/mg of protein/hour. These conditions were similar to those anticipated in subsequent synthesis experiments.

Transfer Reactions

(a) Deoxyadenosine-8-C^{14} (I).--A solution of adenine-8-C^{14} (300 μc, sp. act. 8.5 mc/mmole) and thymidine (42.8 mg, 0.1765 mmole) in 25 ml of 0.1 M phosphate buffer (pH 6.1) was incubated with 1 ml of enzyme solution at 37°C for 2 hours. The solution was overlaid with 5 drops of toluene to prevent bacterial growth. At the end of

the incubation period, the solution was heated in boiling water for 5 minutes to denature the enzyme, cooled in ice, filtered, and chromatographed. The buffer salts, which moved with the solvent front, did not interfere with the complete separation of adenine-8-C^{14} and deoxyadenosine-8-C^{14}. The radioactive, ultraviolet-absorbing bands of residual adenine-8-C^{14} and the deoxyadenosine-8-C^{14} were cut from the large sheets and eluted with dilute ammonium hydroxide. The radiochemical yield of pure product was 91.4%; 2.7% of the initial adenine-8-C^{14} was also recovered. The results are shown in Table II.

Both the thymine and thymidine bands on the chromatogram were radioactive. The total activity in the two bands was 5.62 μc. This finding suggests that the commercial adenine-8-C^{14}, which is obtained from biologically labeled DNA, contained a small amount of C^{14}-thymine.

(b) Deoxyguanosine-8-C^{14} (II).--Guanine-8-C^{14} (300 μc, sp. act. 9.8 mc/mmole) was suspended in 100 ml of de-ionized water and heated in a boiling water-bath to dissolve as much of the guanine as possible. To the resulting suspension at 37°C was added 25 ml of 0.1 M phosphate buffer (pH 6.1), thymidine (37.1 mg, 0.153 mmole), and 1 ml of enzyme solution. A few drops of toluene were added, and the mixture was magnetically stirred in a stoppered flask for 24 hours at 37°C. After 24 hours, some guanine-8-C^{14} was still undissolved; 1 ml of

enzyme solution was added, and incubation was continued an additional 24 hours with stirring. The reaction mixture was heated in boiling water for 7 minutes to coagulate protein and cooled to room temperature. The mixture was then desalted, prior to chromatography, by adsorption of the organic components on a mixture of 80-mesh Fischers' activated charcoal (1 g) and Celite (1 g). The charcoal bed was washed free of phosphate ion with three 10-ml portions of distilled water.* The purines and deoxyribosides were eluted with 50% aqueous ethanol containing 5% pyridine (by volume). The eluate (ca. 1500 ml) was concentrated to ca. 30 ml on a rotary vacuum evaporator at 35°C. The components of the mixture were then separated by chromatography, as described above. The radiochemical yield of pure product was 80%; 7.2% of the initial guanine-8-C^{14} was also recovered. The results are given in Table II.

The thymine and thymidine bands, which were both radioactive, were eluted together and rechromatographed. When the two bands were eluted and assayed, the thymine contained 3.24 μc of activity and the thymidine contained 4.16 μc. As in the adenine-8-C^{14} case above, the guanine-8-C^{14} apparently contained C^{14}-thymine.

(c) Deoxycytidine-2-C^{14} (III).--A solution of cytosine-2-C^{14} (300 μc, sp. act. 5.07 mc/mmole) and deoxyadenosine (74.3 mg, 0.296 mmole) in 25 ml of distilled water was adjusted to pH 5.8 with carbon

*The filtrate and washings contained 3.1 μc of C^{14} activity, which was not absorbed on an additional 1 g of charcoal.

dioxide. One ml of enzyme solution and 5 drops of toluene were added, and the solution was incubated at 37°C for 6 hours. The reaction mixture was then heated in boiling water for 5 minutes, cooled in ice, filtered, and chromatographed as described above. As shown in Table II, it was necessary to treat the residual cytosine-2-C^{14} several times in this manner in order to approach quantitative conversion to the deoxyriboside. The total radiochemical yield of pure product was 83.3%; 11.7% of the initial cytosine-2-C^{14} was also recovered.

The pooled deoxyadenosine from all the deoxycytidine runs contained 5.16 μc of radioactivity, which was probably cytosine-2-C^{14} since the adenine was not active.

(d) Thymidine-2-C^{14} (IV).--A solution of thymine-2-C^{14} (300 μc, sp. act. 5.0 mc/mmole) and deoxyadenosine (75.5 mg, 0.3 mmole) in 25 ml of 0.1 M phosphate buffer (pH 6.1) was incubated with 1 ml of enzyme solution for 2 hours. The reaction mixture was heated, cooled, and desalted, as described for II above, prior to chromatography. As illustrated in Table II, it was necessary to treat the residual thymine-2-C^{14} several times to effect a high degree of conversion to deoxyriboside. In the second thymidine run (Table II), the reaction was run in distilled water, but the pH was 6.7 due to a trace of residual ammonium bicarbonate. This is higher

than the enzyme pH optimum of 5.8 (15), and apparently the 2-hour incubation was too short for equilibrium to be established. Subsequently, the reaction was run 6 hours in distilled water adjusted to pH 5.8-6 with carbon dioxide. The total radiochemical yield of pure product was 74.6%; 9% of the initial thymine-2-C was also recovered.

The pooled deoxyadenosine from all the thymidine runs contained 11.9 μc of radioactivity. This activity is probably all due to thymine-2-C^{14}, since the adenine was free of activity.

DISCUSSION

The trans-N-deoxyribosylase enzyme offers an efficient means of obtaining labeled deoxyribosides, with the natural configuration, providing the labeled bases or a deoxyriboside with the label in the deoxyribose moiety are available. Since the enzyme has no requirement for phosphate, the transfer reaction can be run in neutral aqueous medium or one adjusted with carbon dioxide to the optimum pH of 5.8, thus eliminating the need for a desalting step prior to chromatography.

Although guanine is only slightly soluble in a neutral or slightly acidic (pH 6) aqueous medium, high yields of deoxyguanosine are obtained by stirring a suspension of guanine in thymidine solution for an extended reaction period. Guanine dissolves as the reaction proceeds.

As indicated by the work of Roush and Betz (15), at equilibrium in a reaction mixture containing the enzyme, a purine, a pyrimidine and their deoxyribosides, the reaction rates greatly favor the formation of purine deoxyriboside. Therefore, thymidine is an excellent donor of deoxyribose in the preparation of labeled deoxyadenosine and deoxyguanosine. Comparison of the concentrations of deoxycytidine and thymidine in equilibrium with deoxyadenosine (Table II) and the equilibrium data of Roush and Betz (15) suggests that a pyrimidine deoxyriboside as deoxyribose donor would afford higher yields of labeled thymidine or deoxycytidine than does deoxyadenosine. However, separation of the resulting four-component mixture by paper chromatography or by cellulose column chromatography would be extremely difficult because of the narrow spread in R_f values for most solvents. In any case, it appears that several successive treatments of the pyrimidine bases are required in approaching quantitative conversion to their deoxyribosides.

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TABLE I

Paper chromatography of purine and pyrimidine bases and their deoxyribosides

Compound	R_f Value
Adenine	0.36
Guanine	0.39
Cytosine	0.72
Thymine	0.72
Deoxyadenosine	0.51
Deoxyguanosine	0.62
Deoxycytidine	0.77
Thymidine	0.81

NOTE: Paper, Whatman No. 3. Solvent, 85% saturated ammonium bicarbonate.

TABLE II

Enzymatic synthesis of carbon-14 labeled deoxyribonucleosides

Purine or Pyrimidine						Deoxyriboside			
	C^{14}-Activity						C^{14}-Activity		
Base	Initial μc	Residual μc	Residual % of Initial	Deoxyribose Donor[a]	Time (hours)	Product	Yield μc	% of Initial	% of Recovered[e]
Adenine-8-C^{14}	300	8.1	2.7	t	2	I	274	91.4[b]	97.2
Guanine-8-C^{14}	300	21.6	7.2	t	48	II	240	80.0[c]	91.8
Cytosine-2-C^{14}	300	146.0	49.4	a	6	III	132	44.0	47.5
	146.0	69.7	47.8	a	6		67.8	46.5	49.4
	69.7	35.1	50.4	a	6		33.0	47.3	48.3
	35.1	17.7	50.5	a	2		17.0	48.3	48.9
Thymine-2-C^{14}	300	166.8	55.6	a	2	IV	98.4	32.8[c]	36.6
	166.8	109.0	65.4	a	6		46.2	27.7[d]	29.1
	109.0	68.8	63.2	a	6		38.0	34.9	35.6
	68.8	43.0	62.2	a	6		25.5	37.0	37.2
	43.0	26.9	62.5	a	6		15.7	36.4	36.8

[a]Abbreviations used are: thymidine, t; deoxyadenosine, a.

[b]Phosphate buffer salts not removed prior to chromatography.

[c]Phosphate buffer salts removed prior to chromatography.

[d]Buffer was not used in this and subsequent thymidine runs.

[e]This is % of activity recovered in the residual base and the deoxyriboside formed.

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