ISOTOPIC STUDIES ON 2'-DEOXYINOSINE-8-C¹⁴ FORMATION FROM INOSINE-8-C¹⁴ BY WASHED CELL SUSPENSION OF VITAMIN B¹²-REQUIRING STRAIN OF *LACTOBACILLUS LEICHMANNII*¹

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The authors (1) have reported the formation of deoxyinosine from inosine by cell washed suspension of *Lactobacillus leichmannii* ATCC 7830. Similar studies were further made using inosine-8-C¹⁴ and the washed cell suspension of the strain. First, the isolation and identification of the compounds which appeared in the reaction mixture were tried by two-dimensional paper chromatography and bioautography and also the recovery of the standard compounds added to the reaction mixture. Then, the isolation and determination of 2'-deoxyinosine-8-C¹⁴ were tested similarly, and the metabolic fate of inosine-8-C¹⁴ was examined.

By adding inosine-8-C¹⁴ in the presence of vitamin B₁₂, about 8% of the added C¹⁴ was found in the deoxyinosine fraction by paper chromatoscanning. After addition of hypoxanthine-8-C¹⁴ and D-ribose in the presence of vitamin B₁₂, about 2.7% of the added C¹⁴ was found in the deoxyinosine fraction as detected by paper chromatoscanning, but in case of hypoxanthine and D-ribose-1-C¹⁴ the incorporation into deoxyinosine was scarcely recognized.

EXPERIMENTAL AND RESULTS

1. Two-Dimensional Paper Chromatography of Nucleosides and Related Compounds

Materials — Inosine (HxR), deoxyinosine (dHxR), deoxyadenosine (dAdR), deoxyguanosine (dGuR), deoxycytidine (dCyR), adenosine (AdR), 2'-deoxyribose, D-ribose, adenine (Ad), hypoxanthine (Hx) and cyanocobalamin (CN-B₁₂) were employed.

Method — 300 μg of CN-B₁₂ and 1,000 μg of other materials were dissolved in 1 ml, and 0.01 ml each was spotted on Toyo Filter Paper No. 51 (40×40 cm). At first, one-dimensional ascending paper chromatography was used, developing

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with water-saturated *n*-butanol as solvent I, and in succession, two-dimensional ascending method using 5% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ saturated with isoamyl alcohol or 1 *N* ammonia solution as solvent II (Fig. 1).

FIG. 1 Two-Dimensional Paper Chromatogram of Standard Nucleosides and Related Compounds

Solvent I: water-saturated *n*-butanol; *Rf*: Hx 0.33, HxR 0.15, dHxR 0.28, dGuR 0.22, d-AdR 0.44, dCyR 0.30, AdR 0.30, Ad 0.46, deoxyribose 0.39, D-ribose 0.25, CN-B₁₂ 0.03.

Solvent II: 5% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ satd. with isoamyl alcohol; *Rf*: Hx 0.61, HxR 0.76, dHxR 0.76, dGuR 0.66, dAdR 0.57, dCyR 0.82, AdR 0.56, Ad 0.42, deoxyribose 0.90, D-ribose 0.91, CN-B₁₂ 0.73.

Detection of the Spots—After drying, each spot was preliminarily detected with Manaslu light (short wave 2536 Å, cycle 50). D-Ribose was tested with anilin-phthalic acid reagent by heating at 110° for 10 minutes. Deoxyribose and deoxyribonucleosides were visualized with 5% cysteine-sulfuric acid reagent by heating at 85° for 10 minutes.

Bioautography—Standard two-dimensional paper chromatograms were first prepared with the solution containing each 3 μg of dHxR, dAdR, dGuR, dCyR, HxR, and 0.1 μg of CN-B₁₂ in one spot. Solvents I and II were those described above. Each spot appeared on the paper chromatogram was isolated by cutting.

Each piece was extracted with 2.5 ml of distilled water to which was added 2.5 ml of the assay medium, followed by sterilization for 10 minutes at 15 pounds. CN-B₁₂ and deoxyribonucleosides were estimated by *L. leichmannii* microbioassay. After incubating at 37° for 21 hours, the growth rate was determined at 570 $\text{m}\mu$ using Hitachi spectrophotometer.

UV-Spectra of the Deoxyinosine which Appeared on Paper Chromatograms—Deoxyinosine, 150 μg , was spotted on the paper and was subjected to two-dimensional chromatography. The spot was isolated by cutting, and the portion was extracted with 10 ml of *M*/15 phosphate buffer solution (pH 5.7). After centrifugation for 10 minutes, UV-spectra were estimated with Hitachi spectrophotometer (Fig. 2).

FIG. 2 UV Spectrum of 2'-Deoxyinosine Isolated from the Two-Dimensional Paper Chromatogram Shown in Fig. 1

○, dHxR 15 $\mu\text{g/ml}$; ×, dHxR 15 $\mu\text{g/ml}$.

2. Formation of 2'-Deoxyinosine-8-C¹⁴ from Inosine-8-C¹⁴ and Related Compounds

Microorganism — *Lactobacillus leichmannii* ATCC 7830 was used.

Culture Medium — Stock medium, preculture medium and assay medium were the same as described in the foregoing paper.

Inosine-8-C¹⁴ and Related Compounds — Inosine-8-C¹⁴, with the specific activity of 10 $\mu\text{c}/123 \mu\text{g}$ (Schwarz Bioresearch Incorazgburg), hypoxanthine-8-C¹⁴ with the specific activity of 100 $\mu\text{c}/1,115.7 \mu\text{g}$ (Radiochemical Centre) and D-ribose-1-C¹⁴ with the specific activity of 20.6 $\mu\text{c}/\text{mg}$ (Radiochemical Centre) were employed.

Methods — 1 $\mu\text{c}/\text{ml}$ ($1.1 \times 10^{-4} M$) HxR-8-C¹⁴ was prepared from 10 $\mu\text{c}/123 \mu\text{g}$ HxR-8-C¹⁴ by adding 174.8 μg of HxR. 10 $\mu\text{c}/\text{ml}$ ($8.2 \times 10^{-4} M$) Hx-8-C¹⁴ was prepared

TABLE 1
Reaction System for 2'-Deoxyinosine-8-C¹⁴ Formation from Inosine-8-C¹⁴ and Related Compounds

	Inosine-8-C ¹⁴ 1 $\mu\text{c}/\text{ml}$ ($1.1 \times 10^{-4} M$)	Hypoxanthine-8-C ¹⁴ 10 $\mu\text{c}/\text{ml}$ ($8.2 \times 10^{-4} M$) plus D-ribose ($4 \times 10^{-3} M$)	D-Ribose-1-C ¹⁴ 10 $\mu\text{c}/\text{ml}$ ($3.2 \times 10^{-3} M$) plus hypoxanthine ($2 \times 10^{-3} M$)
	ml	ml	ml
0.1 M $\text{KH}_2\text{PO}_4 \cdot \text{Na}_2\text{HPO}_4$ buffer solution (pH 6.4)	3	3	3
C ¹⁴ -labeled compound	9	2.44	1.24
Addition	—	1	1
Water	1.5	7.06	8.26
5 mg/ml <i>L. leichmannii</i> washed cell suspension	1.5	1.5	1.5
Total	15.0	15.0	15.0

from 100 μc /1,115.7 μg of Hx-8- C^{14} by diluting with water. 10 μc /ml ($3.2 \times 10^{-3}M$) D-ribose-1- C^{14} was likewise prepared from 20.6 μc /mg of D-ribose-1- C^{14} .

The reaction system was given in Table 1. Each component except for washed cell suspension was sterilized separately for 10 minutes at 15 pounds. To the mixture of each component was added 5 mg/ml of washed cell suspension, and the whole was incubated at 37° for 20 hours. After cooling to 0°, the reaction mixture was centrifuged at 4,000 rpm for 30 minutes. The cells were washed twice with 2 ml of saline, the supernatant was made up to 20 ml with saline, and the compounds in the supernatant were estimated by two-dimensional paper chromatography, autoradiography and paper chromatoscanning. The results are shown in Figs. 3—8 and in Table 2.

FIG. 3 Two-Dimensional Paper Chromatogram and Autoradiogram of 2'-Deoxyinosine-8- C^{14} Formed from Inosine-8- C^{14}

A, two-dimensional paper chromatogram; B, autoradiogram. Reaction products—Solvent I: water-saturated *n*-butanol; *Rf*: Hx 0.50, dHxR 0.42, dGuR 0.38, dAdR 0.62, Ad 0.66, deoxyribose 0.32, D-ribose 0.19; Solvent II: 5% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ satd. with isoamyl alcohol; *Rf*: Hx 0.60, HxR —, dHxR 0.75, dGuR 0.66, dAdR 0.57, Ad 0.40, deoxyribose 0.88, D-ribose 0.93.

Standard compounds—Solvent I: water-saturated *n*-butanol; *Rf*: Hx 0.54, HxR 0.28, dHxR 0.44, dGuR 0.36, dAdR 0.61, Ad 0.65, deoxyribose 0.37, D-ribose 0.20; Solvent II: 5% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ satd. with isoamyl alcohol; *Rf*: Hx 0.59, HxR 0.74, dHxR 0.75, dGuR 0.66, dAdR 0.58, Ad 0.42, deoxyribose 0.90, D-ribose 0.91.

TABLE 2

Amount of 2'-Deoxyinosine-8- C^{14} Formed in the Reaction Mixture after 20-Hour Incubation

	Inosine-8- C^{14}	Hypoxanthine-8- C^{14} plus D-ribose	D-Ribose-1- C^{14} plus hypoxanthine
	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>
Initial amount of C^{14}	393,507	3,543,124	1,091,293
Amount of C^{14} in 2'-dHxR-8- C^{14}	31,292	95,361	1,129
Amount of 2'-dHxR-8- C^{14} formed	8.0%	2.7%	0.1%

FIG. 4 Paper Chromatoscanning of 2'-Deoxyinosine-8-C¹⁴ which Appeared in Two-Dimensional Paper Chromatogram Shown in Fig. 3

Sample: Inosine-8-C¹⁴, 9 μ c/268 μ g/9 ml. Two-dimensional paper chromatography, Solvent I, water-saturated *n*-butanol; Solvent II, Na₂HPO₄·12H₂O saturated with isoamyl alcohol. Range, 3,000 cpm; slit, 5 mm; velocity, 50 mm/min; volt, 1,200; sec, 0.3.

SUMMARY

1. Isolation and confirmation of 2'-deoxyinosine and related compounds which appeared in the reaction mixture were made by two-dimensional ascending paper chromatography, and each spot was applied to bioautography, autoradiography and chromatoscanning. The recoveries of the standard compounds previously added to the reaction mixture were satisfactory.

2. Formation of 2'-deoxyinosine-8-C¹⁴ from inosine-8-C¹⁴ in the presence of vitamin B₁₂ was tested by two-dimensional paper chromatography, and paper chromatoscanning, and the labeled deoxyinosine was detected in the amount of 8% of the added C¹⁴.

3. Using the similar method, the incorporation of the labeled carbon of hypoxanthine-8-C¹⁴ or D-ribose-1-C¹⁴ in deoxyinosine was studied. In the presence of hypoxanthine-8-C¹⁴, D-ribose and CN-B₁₂, the labeled carbon was found in the deoxyinosine fraction by paper chromatoscanning in the amount of 2.7% of the added C¹⁴, but in the presence of hypoxanthine, D-ribose-1-C¹⁴ and CN-B₁₂, the incorporation of the labeled carbon into deoxyinosine was scarcely detected.

FIG. 5 Two-Dimensional Paper Chromatogram and Autoradiogram of 2'-Deoxyinosine-8- C^{14} Formed in the Presence of Hypoxanthine-8- C^{14} and D-Ribose

A, two-dimensional paper chromatogram; B, autoradiogram. Reaction products—Solvent I: water-saturated *n*-butanol; *Rf*: Hx 0.21, HxR 0.05, dHxR 0.15, dGuR 0.14, dAdR 0.30, Ad 0.30, deoxyribose 0.34 D-ribose 0.15; Solvent II: 5% $Na_2HPO_4 \cdot 12H_2O$ satd. with isoamyl alcohol; *Rf*: Hx 0.56, HxR 0.75, dHxR 0.76, dGuR 0.65, dAdR 0.58, Ad 0.39, deoxyribose 0.96, D-ribose 0.95.

Standard compounds—Solvent I: water-saturated *n*-butanol; *Rf*: Hx 0.20, HxR 0.05, dHxR 0.15, dGuR 0.14, dAdR 0.36, Ad 0.28, deoxyribose 0.35, D-ribose 0.15; Solvent II: 5% $Na_2HPO_4 \cdot 12H_2O$ satd. with isoamyl alcohol; *Rf*: Hx 0.60, HxR 0.79, dHxR 0.79, dGuR 0.68, dAdR 0.60, Ad 0.41, deoxyribose 0.95, D-ribose 0.95.

FIG. 6 Paper Chromatoscanning of 2'-Deoxyinosine-8- C^{14} which Appeared in Two-Dimensional Paper Chromatogram Shown in Fig. 5

Sample: hypoxanthine-8- C^{14} , 9.792 μc /108.8 μg /8 ml + unlabeled ribose 240 μg . Two-dimensional paper chromatography. Solvent I, water-saturated *n*-butanol. Solvent II, 5% $Na_2HPO_4 \cdot 12H_2O$ saturated with isoamyl alcohol. Range, 3,000 cpm; slit, 2 mm; velocity, 25 mm/min; volt, 1,200; sec., 0.3.

FIG. 7 Two-Dimensional Paper Chromatogram of 2'-Deoxyinosine-8- C^{14} Formed in the Presence of Hypoxanthine and D-Ribose-1- C^{14}

Reaction products — Solvent I: water-saturated *n*-butanol; *Rf*: Hx 0.20, HxR 0.03, dHxR 0.12, dGuR 0.10, dAdR 0.21, Ad 0.25, deoxyribose 0.25, D-ribose 0.13; Solvent II: 5% $Na_2HPO_4 \cdot 12H_2O$ satd. with isoamyl alcohol; *Rf*: Hx 0.60, HxR 0.78, dHxR 0.76, dGuR 0.64, dAdR 0.55, Ad 0.41, deoxyribose 0.92, D-ribose 0.95.

Standard compounds — Solvent I: water-saturated *n*-butanol; *Rf*: Hx 0.24, HxR 0.06, dHxR 0.12, dGuR 0.11, dAdR 0.22, Ad 0.26, deoxyribose 0.27, D-ribose 0.14; Solvent II: 5% $Na_2HPO_4 \cdot 12H_2O$ satd. with isoamyl alcohol; *Rf*: Hx 0.57, HxR 0.77, dHxR 0.77, dGuR 0.67, dAdR 0.55, Ad 0.42, deoxyribose 0.93, D-ribose 0.96.

FIG. 8 Paper Chromatoscanning of 2'-Deoxyinosine-8- C^{14} which Appeared in Two-Dimensional Paper Chromatogram Shown in Fig. 7

Sample, ribose-1- C^{14} , 4,944 μ c/240 μ g/8 ml + unlabeled hypoxanthine 108.8 μ g. Two-dimensional paper chromatography, Solvent I, water-saturated *n*-butanol; Solvent II, 5% $Na_2HPO_4 \cdot 12H_2O$ saturated with isoamyl alcohol. Range, 300 cpm; slit, 5 mm; velocity, 300 mm/hr; volt, 1,200; sec., 0.3.

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