Studies on the Biosynthesis of Kanamycins

Part II. Incorporation of the Radioactive Degradation Products of Kanamycin A or Related Metabolites into Kanamycin A

By Michio KOJIMA, Yujiro YAMADA and Hamao UMEZAWA*

Research Laboratories of Meiji Seika Kaisha, Ltd., Yokohama

Received March 8, 1969

Incorporation of the radioactive degradation products of kanamycin A or related metabolites into kanamycin A by growing cells of *Streptomyces kanamyceticus* was examined. ^3H -Deoxystreptamine was incorporated into deoxystreptamine moiety of kanamycin, but neither ^{14}C -3-amino-3-deoxy-D-glucose nor ^{14}C -6-amino-6-deoxy-D-glucose was incorporated. ^3H -Kanamycin A added to medium was modified and inactivated.

Biosynthesis of kanamycin A from ^{14}C -D-glucose and ^{14}C -D-glucosamine added at various phases of the shaking culture of *Streptomyces kanamyceticus* has been studied and reported in a previous paper.¹⁾ ^{14}C -D-Glucosamine is incorporated into kanamycin A at much higher rate than ^{14}C -D-glucose and the incorporated radioactivity is found exclusively in 6-amino-6-deoxy-D-glucose moiety. As reported in the same paper, paromamine is produced by the same organism and ^{14}C -D-glucosamine is incorporated into D-glucosamine moiety of this compound.

In order to study the biosynthetic pathway of kanamycin A, it was examined whether its degradation products, such as deoxystreptamine, 6-amino-6-deoxy-D-glucose and 3-amino-3-deoxy-D-glucose, were utilized for biosynthesis of kanamycin A. The incorporation of paromamine into kanamycin A and the fate of kanamycins A and B added to the cultured medium of *S. kanamyceticus* were also studied. Results of these experiments are presented in this paper.

MATERIALS AND METHODS

Culture techniques. *Streptomyces kanamyceticus* 205 was cultivated by the same procedure as described in a previous paper¹⁾ and ^{14}C - or ^3H -compounds were added at the time as indicated in the tables.

Labeled compounds. ^3H -Deoxystreptamine (20 $\mu\text{Ci}/\text{mg}$) was prepared by Dr. T. Komai, National Institute of Health, Japan, by tritiating deoxystreptamine and generously supplied to the authors. ^{14}C -(U)-3-Amino-3-deoxy-D-glucose²⁾ (0.01 $\mu\text{Ci}/\text{mg}$) was obtained from Prof. S. Umezawa, Institute of Technology, Keio University. ^3H -Kanamycin A (2.3 $\mu\text{Ci}/\text{mg}$) was obtained from Dr. K. Umemura of our Laboratories. ^{14}C -6-Amino-6-deoxy-D-glucose was obtained by acid hydrolysis of ^{14}C -kanamycin A. ^{14}C -Paromamine and ^{14}C -kanamycin B were prepared by cultivating *S. kanamyceticus* in the presence of 1- ^{14}C -D-glucosamine.

Assay of radioactivity. The radioactivity of spots on a developed paperchromatogram was detected by the radioautogram using X-ray film. Other radio-methological methods employed were the same as described in the previous paper.¹⁾

* Institute of Microbial Chemistry.

1) M. Kojima, Y. Yamada and H. Umezawa, *Agr. Biol. Chem.*, **32**, 467 (1968).

2) S. Umezawa, K. Umino, S. Shibahara, M. Hamada and S. Omoto, *J. Antib.*, **20A**, 355 (1967).

RESULTS AND DISCUSSION

Separation of kanamycins A, B, C and paromamine in the cultured broth

The cultured broth of *S. kanamyceticus* obtained by the shaking culture was subjected to a chromatography on Amberlite IRC 50 (Na type). The crude kanamycin eluted with 1N ammonium hydroxide was subjected to Dowex 1×2 (OH type) resin chromatography described by Rothrock *et al.*³¹ and, as shown in Fig. 1, separated into three fractions. The first two peaks, P-I and P-II, were not completely separated and P-III showed an independent peak. Each fraction obtained by the chromatography was paperchromatographed using *n*-butanol-pyridine-acetic acid-water (6:4:1:3 by volume) at 20~25°C for 5 days. After development, the filter paper was placed on Fuji X-ray film in a dark room for 20 hr and

FIG. 1. Chromatography of Crude Kanamycin on Dowex 1×2.

Crude kanamycin (50 mg, 11.5 mg as kanamycin A, *ca.* 5×10^6 dpm) is applied to the column of Dowex 1×2 (OH type, 200~400 mesh) (8×1200 mm) and eluted with distilled water. Paper discs (8 mm diameter) were dipped into each fraction and dried on filter paper. Bioactivity and radioactivity of the fractions were examined using these paper discs.

Total counts of each peak was as follows; PI 4.0×10^5 dpm, PII 3.0×10^5 dpm, and PIII 1.7×10^5 dpm (5.5 mg as kanamycin A).

3) J. W. Rothrock, R. T. Goegelman and F. J. Wolf, *Antibiotics Annual*, 1958~1959, p. 796, Medical Encyclopedia, Inc. N.Y.

FIG. 2. Radioautogram of Kanamycin Derivatives Obtained from Cultured Broth.

Each radioactive peak (PI~PIII, *ca.* 10^5 dpm) was concentrated *in vacuo* and paperchromatographed using *n*-butanol-pyridine-acetic acid-water (6:4:1:3 by volume) as solvent system for 5 days at 20~25°C. After development, the filter paper was placed on Fuji X-ray film 200 (25.4×30.5 cm) in the dark for 20 hr and film was developed. Paperchromatogram of the authentic samples of kanamycin derivatives was developed by ninhydrin spray.

the film was developed. As shown in Fig. 2, kanamycins A, B, C, and paromamine were effectively separated. Purification of those compounds was carried out by eluting them with distilled water as described by Consden *et al.*⁴⁾ for the purification of small peptides. The data shown in Figs. 1 and 2 were obtained with the cultured broth containing 1-¹⁴C-D-glucosamine. After incubation for 3 days, 1-¹⁴C-D-glucosamine (0.6 μ Ci/ml) was added to 50 ml of culture broth which was further incubated for 2 days. Figures 1 and 2 indicate that 1-¹⁴C-D-glucosamine is incorporated into kanamycins A, B, C, and paro-

4) R. Consden, A. H. Gardon and A. J. P. Martin, *Biochem. J.*, **41**, 590 (1947).

amine. Figure 2 also shows the presence of unidentified radioactive compound biosynthesized from 1- ^{14}C -D-glucosamine. As reported in the previous paper,¹¹ D-glucosamine must be taken into 2- or 6-amino 2- or 6-deoxy-D-glucose or 2,6-diamino-2,6-dideoxy-D-glucose.

Incorporation of ^{14}C - or ^3H -degradation products of kanamycin A, ^{14}C -paromamine and ^{14}C -kanamycin B into kanamycin A.

^{14}C -6-Amino-6-deoxy-D-glucose, ^{14}C -3-amino-3-deoxy-D-glucose, ^3H -deoxystreptamine, ^{14}C -paromamine and ^{14}C -kanamycin B were added in the later phase of the shaking culture and incorporation into kanamycins and paromamine were examined. Table I shows the incorporation rate of these compounds into kanamycin A. ^{14}C -6-Amino-6-deoxy-D-glucose, ^{14}C -3-amino-3-deoxy-D-glucose, ^{14}C -paromamine and ^{14}C -kanamycin B were not incorporated

into kanamycin A. However, ^3H -deoxystreptamine was incorporated. Incorporation of the compounds, except deoxystreptamine, into kanamycins B, C, and paromamine was not also observed. ^{14}C -Paromamine and ^{14}C -kanamycin B were recovered unchanged.

The fact that ^{14}C -6-amino-6-deoxy-D-glucose and ^{14}C -3-amino-3-deoxy-D-glucose were not incorporated into kanamycin A might suggest that these aminosugars were not taken into cells or not utilized for biosynthesis of kanamycin A. It is possible that these aminosugars are utilized in their nucleoside diphosphate form for biosynthesis of kanamycin A.

Incorporation of ^3H -deoxystreptamine into kanamycin A

As shown in Table I, deoxystreptamine was incorporated into kanamycin A. In order to know the mechanism in detail, the incorporation of ^3H -deoxystreptamine which was

TABLE I. INCORPORATION OF ^{14}C - OR ^3H -DEGRADATION PRODUCTS OF KANAMYCIN A, ^{14}C -PAROMAMINE AND ^{14}C -KANAMYCIN B INTO KANAMYCIN A

Labeled compounds	Added at	dpm/ml	S. A. of KMA dpm/m μ mol
^{14}C -3-Amino-3-deoxy-D-glucose	3 days	4.0×10^3	<0.05
^{14}C -6-Amino-6-deoxy-D-glucose	3 days	1.6×10^3	<0.05
^3H -Deoxystreptamine	3 days	5.0×10^5	58.1
^{14}C -Kanamycin B	3 days	1.1×10^3	<0.05
^{14}C -Paromamine	4 days	1.5×10^3	<0.05

Labeled degradation products of kanamycin A or related compounds were added in the later phase of shaking culture and at 5 days after the inoculation, the culture was stopped and analyzed.

TABLE II. EFFECT OF THE TIME OF ADDITION OF ^3H -DEOXYSTREPTAMINE ON ITS INCORPORATION INTO KANAMYCIN A

Exp. No.	^3H -Deoxystreptamine added (2.6×10^7 dpm) at days	Kanamycin synthesized			^3H -Incorporated %
		Amount* μ mol	S. A. of KMA dpm/m μ mol	Total activity** dpm $\times 10^{-6}$	
1	0	14.5	231	3.4	13.0
2	1	15.7	178	3.2	12.3
3	2	12.8	168	2.3	8.9
4	3	14.1	93	1.3	5.0
5	4	12.3	75	0.9	3.5

* Total amount in 10 ml of cultured broth. (1.0 μ mol = 484 μ g free base)

** All bioactivities of the cultured broth were calculated as kanamycin A.

TABLE III. INACTIVATION OF ^3H -KANAMYCIN A ADDED IN THE COURSE OF SHAKING CULTURE

Exp. No.	^3H added at days	Cultured broth		Kanamycin A obtained		
		Total counts $\text{dpm} \times 10^{-6}$	Kanamycin μmol	S. A.** $\text{dpm}/\text{m}\mu\text{mol}$	Recovered $\text{dpm} \times 10^{-5}$	%
1	2	1.08	11.2	34.8	3.9	40
2	3	0.92	10.6	37.8	3.9	40
3	4	0.86	9.3	60.4	5.6	61
4*	5	1.07	12.4	75.2	9.2	100

^3H -Kanamycin A (*ca.* 10^6 dpm, $0.45 \mu\text{mol}$) was added to 10 ml of shaking culture broth at various phases as indicated in the Table.

In this series of experiments (Exp. No. 1~4), practically all bioactivities were due to kanamycin A.

* ^3H -Kanamycin A was added just before the cessation of the shaking culture.

** S. A. (Specific activity) of kanamycin A was calculated after separation by Dowex 1 chromatography.

added at various cultural phases was examined. As shown in Table II, maximal incorporation was obtained when it was added at the early phases of the shaking culture. These results suggest that deoxystreptamine is taken into cells and this compound or its derivative participates in biosynthesis of deoxystreptamine moiety of kanamycins.

Distribution of ^3H among the three moieties of kanamycin A molecule was studied by the method as described in the previous paper.¹⁾ As shown in Fig. 3, practically all radioactivity was found in the deoxystreptamine moiety.

myo-Inositol is known to be utilized for biosynthesis of deoxystreptamine and the mode of incorporation of deoxystreptamine is of special interest in relation to *myo*-inositol incorporation.

Inactivation of ^3H -kanamycin A added at various cultural phases

^3H -Kanamycin A was added at various shaking cultural phases and ^3H distribution among the fractions obtained from Dowex 1 chromatography and the specific activity of resulted kanamycin A were examined. Results are shown in Table III. Practically all radioactivities remained in kanamycin A fraction when ^3H -kanamycin A was added just before the cessation of the shaking culture. The total radioactivity of ^3H -kanamycin A added was not entirely recovered in resulted kanamycin A fraction and the total radioactivity of

FIG. 3. Distribution of ^3H Derived from ^3H -Deoxystreptamine in the Three Moieties of Kanamycin A.

^3H -Deoxystreptamine was added at 1, the time of inoculation; 2, at 1 day; 3, at 2 days; 4, at 3 days; 5, at 4 days after inoculation, respectively. All cultures were stopped at 5 days after inoculation and kanamycin A fractions were collected by Dowex 1 chromatography and hydrolyzed with 6N HCl for 60 min in boiling water. Solvent system for paperchromatography was *n*-butanol-acetic acid-water (4:2:1 by volume) at 20~25°C for 30 hr.

DS: deoxystreptamine.

6AG: 6-amino-6-deoxy-D-glucose.

3AG: 3-amino-3-deoxy-D-glucose.

Experimental number (Exp. 1~5) is the same as in Table II.

the cultured broth filtrate was not significantly reduced during the shaking culture. This is of special interest, because the decrease in production of kanamycin A is often observed after the production has reached to its maximum.

Acknowledgement. The authors wish to express their sincere thanks to Dr. T. Komai, National Institute of Health, Japan, for the preparation of tritiated compound and also to Prof. S. Umezawa, Institute of Technology, Keio University, for the gift of ^{14}C -3-amino-3-deoxy-D-glucose.