ISOLATION OF KANAMYCIN AND PAROMAMINE INACTIVATED BY *E. COLI* CARRYING R FACTOR

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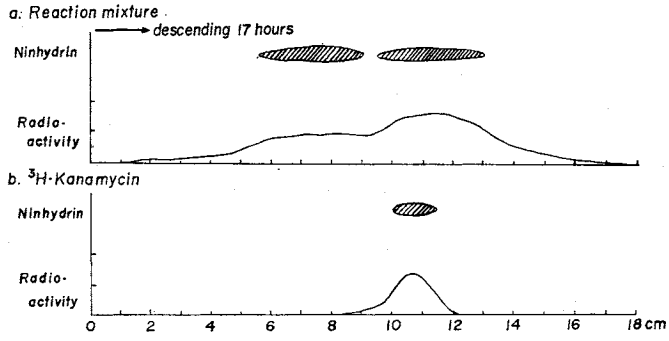
Kanamycin and paromamine were inactivated in the presence of ATP and Mg^{++} by an enzyme solution obtained from *E. coli* carrying R factor. The inactivated kanamycin was isolated and it was found that the hydroxyl group on C-3 of 6-amino-6-deoxy-D-glucose moiety was phosphorylated. In the inactivated paromamine, the hydroxyl group on C-3 of D-glucosamine moiety was phosphorylated. Besides the inactivated kanamycin above described, another inactivated kanamycin containing phosphate was isolated, though it is not certain that this is the product of enzymatic inactivation.

The effect of an enzyme solution prepared from *E. coli* carrying R factor on kanamycin, paromamine and dihydrostreptomycin has been studied. These antibiotics are inactivated in the presence of ATP and Mg^{++}[1]. The requirement of the inactivation reaction for ATP suggests that these antibiotics would be phosphorylated and inactivated. In this paper, the isolation of the inactivated kanamycin and paromamine is reported.

The enzyme solution was prepared from *E. coli* K 12 ML 1629 which was obtained by transmission of R factor from a naturally isolated resistant *E. coli* to *E. coli* K 12 ML 1410 which had been made resistant to nalidixic acid. The procedure of the preparation of the enzyme and the inactivation reaction are described in another paper[1].

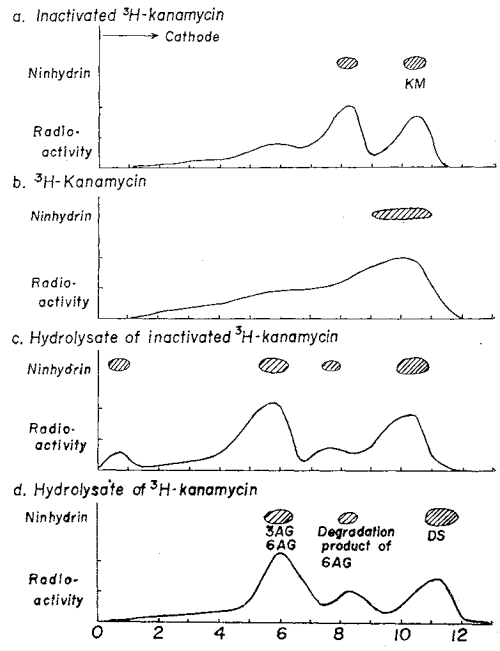
The reaction mixture containing the inactivated ^{3}H-kanamycin gives two radioactive peaks on paper chromatography as shown in Fig. 1. These peaks are positive to ninhydrin and one of them is ^{3}H-kanamycin. Another peak corresponding to the inactivated ^{3}H-kanamycin appears at lower Rf than kanamycin. A light brown powder isolated from the reaction mixture by Amberlite IRC-50 resin contains 100% of the radioactivity of ^{3}H-kanamycin added and shows also

Fig. 1. Paper chromatography of reaction mixture after the phosphorylative inactivation and ^{3}H-kanamycin (solvent : PrOH-Pyr.-AcOH-H_2O ; 15 : 10 : 3 : 12, v/v)



two radioactive peaks on a high-voltage paper electrophoresis as shown in Fig. 2. Besides these two main peaks, a broad low peak which is negative to ninhydrin is shown in Fig. 2-a, but as shown in Fig. 2-b, this small peak is observed also in ^{3}H-kanamycin employed. This small peak is, thus, a contaminating substance in ^{3}H-kanamycin. The position of the inactivated ^{3}H-kanamycin on the high-voltage paper electrophoresis is utilized to detect the inactivated kanamycin in extraction and purification fractions. After the inactivation reaction, the reaction mixture is heated at 85°C for 5 minutes and diluted about six times with distilled water. The inactivated kanamycin in the diluted solution is adsorbed on Amberlite IRC-50 resin in the sodium form and eluted with 1% aqueous ammonia. The ninhydrin-positive eluate containing the inactivated kanamycin is concentrated under reduced pressure and subjected to column chromatography on Dowex 1 X 2 with 0.5 N HCl for the elution. Two inactivated kanamycins are isolated by the column chromatography of Amberlite CG-50 in ammonium form. The one designated inactivated kanamycin-II appears in the aqueous effluent. Another one designated inactivated kanamycin-I is adsorbed on the resin and appears when eluted with 1% aqueous ammonia. A minute amount of the inactivated kanamycin-II is found in the solution passed through Amberlite IRC-50 resin and in the water wash, when the reaction mixture is passed over a column of Amberlite IRC-50 in sodium form.

Fig. 2. High-voltage paper electrophoresis of ^{3}H-kanamycin, inactivated ^{3}H-kanamycin and their hydrolyzates (AcOH-HCOOH-H_2O; 75 : 25 : 900, 3,500 V, 15 min.)



The inactivated kanamycin-I migrates like inactivated ^{3}H-kanamycin on electrophoresis and has the following properties: darkening at 270~280°C but no melting at 300°C. Anal.: Calcd. for $C_{18}H_{35}N_4O_{11}\cdot PO(OH)_2\cdot 2H_2O$, C 36.00, H 6.88, N 9.33, O 42.63, P 5.16; found, C 35.26, H 6.83, N 9.08, O 41.76, P 5.20; positive ninhydrin and positive for phosphorous test by HANES reagent[2].

The inactivated kanamycin-I is a monophosphorylated kanamycin as shown by the analytical results and by the behavior on the paper electrophoresis. Under high-voltage paper electrophoresis using acetic acid : formic acid : water (75 : 25 : 900, v/v) under 3,500 V for 15 minutes, it moves toward the cathode 10.4~11.5 cm. This behavior on the electrophoresis is the same as that of kanamycin-6′-phosphate which has been synthesized by S. UMEZAWA *et al.*[3] Kanamycin moves toward the cathode 13.1~15.4 cm and thus the inactivated kanamycin-I is less basic than kanamycin. The inactivated kanamycin-I is completely converted to kanamycin by heating in

Table 1. Reactivation of the inactivated kanamycin-I in 0.4 M $HClO_4$ with or without adjustment of pH at 80~83°C

pH adjusted	Hours	% activated*
no	1	<1.3
no	8	1.3
4.0	1	3.0
4.0	3	6.3
4.0	8	16.3
4.0	20	80.0
6.8	1	<1.3
6.8	8	1.6

* Theoretical % is 80.7.

0.4 M $HClO_4$ adjusted to pH 4.0 at 80~83°C as shown in Table 1. Refluxing in hydrochloric acid gives slight or no reactivation of the inactivated kanamycin-I. As reported in another paper[1], it is converted to kanamycin by alkaline phosphatase of calf mucosa. The inactivated kanamycin-I consumed 2.0 moles of periodate at pH 4.05 in 24 hours, while kanamycin consumed 3.8 moles. The acid hydrolysis of the periodate-oxidized product from inactivated kanamycin-I gives deoxystreptamine and 6-amino-6-deoxy-D-glucose but not 3-amino-3-deoxy-D-glucose, although kanamycin gives only deoxystreptamine. Four amino nitrogens are shown by VAN SLYKE method in both kanamycin and the inactivated kanamycin-I. Deamination of the inactivated kanamycin-I followed by hydrolysis gives no ninhydrin positive product. The structure shown below can be assigned on the following basis: one mole of phosphoric acid in the molecule by analysis, the conversion to kanamycin by heating in $HClO_4$ at pH 4.0 or by alkaline phosphatase, monophosphorylated behavior on paper electrophoresis, 4 amino nitrogens, consumption of 2 moles of periodate and presence of 6-amino-6-deoxy-D-glucose after periodate oxidation followed by the acid hydrolysis.

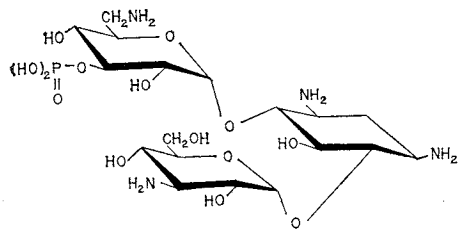


Table 2. Thin-layer chromatography of kanamycin (KM), inactivated kanamycins (IKM-I, IKM-II) and their hydrolyzates using Silica gel G.

Solvent	BuOH-AcOH-H_2O (4 : 2 : 1)				PrOH-Pyr.-AcOH-H_2O (51 : 20 : 6 : 24)					
Rf	0	0.06 (DS)	0.18 (6AG)	0.28 (3AG)	0.03 (IKM)	0.17	0.29~0.44 (KM)	0.56 (DS)	0.64 (6AG)	0.71 (3AG)
Kanamycin	+	−	−	−	−	−	+	−	−	−
Inactivated kanamycin-I	+	−	−	−	+	−	−	−	−	−
Inactivated kanamycin-II	+	−	−	−	+	−	−	−	−	−
Acid hydrolyzate of KM	−	+	+	+	−	−	−	+	+	+
Acid hydrolyzate of IKM-I	+	+	+	+	−	+	−	+	+	+
Acid hydrolyzate of IKM-II	+	+	+	+	−	+	−	+	+	+
Acid hydrolyzate of periodate-oxidized IKM-I	+	+	+	−						
Acid hydrolyzate of periodate-oxidized IKM-II	+	+	+	−						

DS : deoxystreptamine, 6AG : 6-amino-6-deoxy-D-glucose, 3AG : 3-amino-3-deoxy-D-glucose.

The inactivated kanamycin-II has the following properties: darkening at 250~260°C but no melting at 300°C. Anal: found, C 33.73, H 6.45, N 10.89, N 42.36, P 4.90. It gives positive ninhydrin and positive phosphorous tests by HANES reagent,

and shows less basic behavior on electrophoresis than the inactivated kanamycin-I. The inactivated kanamycin-II moves toward the cathode 7.5~8.6 cm. It is not converted to kanamycin by heating in 6 N HCl for 30 minutes at 100°C. Two moles of periodate are consumed, if the molecular weight of the inactivated kanamycin-II is assumed to be 600, and the subsequent hydrolysis gives deoxystreptamine and 6-amino-6-deoxy-D-glucose but not 3-amino-3-deoxy-D-glucose.

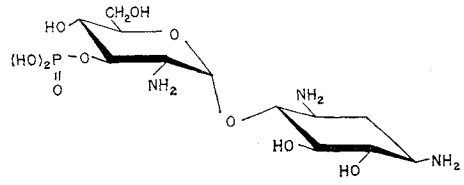
Results of thin-layer chromatography of the inactivated kanamycin-I, the inactivated kanamycin-II, their acid hydrolyzates with or without periodate oxidation, and kanamycin are shown in Table 2. As is well known, the hydrolysis of kanamycin under reflux in 6 N HCl for 30 minutes gives deoxystreptamine, 6-amino-6-deoxy-D-glucose, 3-amino-3-deoxy-D-glucose and a degradation product of 6-amino-6-deoxy-D-glucose. Besides these hydrolysis products, both inactivated kanamycins give an unknown compound with have Rf 0 in the solvent system *n*-butanol : acetic acid : water (4 : 2 : 1, v/v) and Rf 0.17 in the system *n*-propanol : pyridine : acetic acid : water (51 : 20 : 6 : 24, v/v). This unidentified hydrolysis product gives positive ninhydrin and positive phosphorous tests and is also observed in the acid hydrolyzate of the periodate-oxidized inactivated kanamycins. Hydrolysis of organic phosphate has been studied with simple compounds and it has been noted that hydrolysis with hydrochloric acid gives complex products because of the strong uncleophilicity of chloride ion[4]. The inactivated kanamycin-I is completely converted to kanamycin by heating at 80°C in 0.4 M $HClO_4$ at pH 4.0 for 20 hours, but less than 1 % is converted to kanamycin by heating at 100°C in 0.5~1 N HCl for 30 minutes, 1 hour or 8 hours. The conversion to kanamycin is not observed with alkaline hydrolysis. Only 3.6 % of the inactivated kanamycin-II is converted to kanamycin by heating in 0.4 M $HClO_4$ at pH 4.0 and 80~83°C for 20 hours. The hydrolysis of inactivated kanamycins in thus complex and it is necessary to have more informations on hydrolysis of organic phosphates in order to explain the hydrolysis results with inactivated kanamycins.

Inactivated kanamycin-II is not shown by paper electrophoresis of the reaction mixture containing ^3H-kanamycin after enzymatic inactivation, and is found only in traces in the aqueous effluent of the reaction mixture through Amberlite IRC-50 resin. It can be isolated after further purification by anion resin chromatography using hydrochloric acid for elution and Amberlite CG-50 resin chromatography using aqueous ammonia for elution. It is not certain that the inactivated kanamycin-II is a product of enzymatic inactivation.

The enzyme solution which inactivates kanamycin inactivates also paromamine. The inactivated paromamine is adsorbed on Amberlite IRC-50 resin in the sodium form, and eluted with 1 % NH_4OH. The inactivated paromamine in the ninhydrin positive eluate is subjected to chromatography on Amberlite CG-50 resin in ammonium form and appears in the aqueous effluent. The ninhydrin positive effluent is concentrated under reduced pressure and the inactivated paromamine is crystallized by the addition of methanol.

The inactivated paromamine darkens at 260~290°C but does not melt at 300°C. Anal : calcd. for $C_{12}H_{24}N_3O_7 \cdot PO(OH)_2 \cdot 2H_2O$, C 32.80, H 6.88, N 9.56, O 43.70, P 7.05;

found, C 32.26, H 6.72, N 8.94, O 42.24, P 7.80. It gives a positive ninhydrin test and moves toward the cathode 7.8~9.8 cm on the high-voltage paper electrophoresis using acetic acid : formic acid : water (75 : 25 : 900, v/v). Paromamine moves 12.5~15.0 cm. Hydrolysis under reflux in 6 N HCl for 2 hours gives deoxystreptamine, D-glucosamine and an unidentified compound which contains phosphorous and gives a positive ninhydrin test on paper electrophoresis. As described above in the case of inactivated kanamycins, an identified compound containing phosphorous is observed in the acid hydrolyzate. The inactivated paromamine is converted to paromamine by heating in 0.4 M $HClO_4$ at pH 4.0 for 20 hours or by alkaline phosphatase. After this reaction, the unidentified compound is not observed. Paromamine consumed 4.4 moles of periodate and the inactivated paromamine consumed 1.6 moles at pH 4.05 for 24 hours. Acid hydrolysate after periodate oxidation of the inactivated paromamine shows the presence of D-glucosamine. On the basis of these results, the structure shown below can be assigned to the inactivated paromamine.



Experimental

1. Extraction and electrophoresis of the inactivated ^{3}H-kanamycin : The inactivation reaction was run at 37°C for 96 hours in the following solution : ^{3}H-kanamycin monosulfate 24 mg (36 μ moles), disodium ATP 144 mg (238 μ moles), 0.5 M phosphate buffer (pH 7.8) 1.8 ml, 0.07 M $MgCl_2$—1.1 M KCl solution 0.3 ml, the enzyme solution from *E. coli* K 12 ML 1629 0.9 ml. After the reaction, it was heated at 80°C for 5 minutes and filtered. The filtrate (2.5 ml) was diluted to 20 ml with distilled water and pH was 7.0. An aliquot of the filtrate (0.006 ml) was spotted on paper (Toyo No. 51, 2×40 cm) and was subjected to descending paper chromatography using *n*-propanol : pyridine : acetic acid : water (15 : 10 : 3 : 12 v/v) for 17 hours. Then, as shown in Fig. 1, it showed two radioactive peaks which gave positive ninhydrin tests, one of which was identified as ^{3}H-kanamycin. The other peak was thus determined to be the inactivated ^{3}H-kanamycin.

The filtrate was passed through a coulmn of Amberlite IRC-50 (20 ml of Na form, pH 6.8) and the column was washed with 50 ml of distilled water. The effluent showed substantially no radioactivity. The column was eluted with 1 % NH_4OH. Then, the radioactivity appeared in the ninhydrin positive fractions. The ninhydrin-positive fractions were combined, evaporated under reduced pressure and dried to give 35 mg of a light brownish powder. Its behavior on high-voltage paper electrophoresis is shown in Fig. 2.

2. Isolation of inactivated kanamycins : The inactivation reaction was run in the following solution at 37°C, for 20 hours ; kanamycin sulfate 240 mg (0.4 m mole), disodium ATP 1,500 mg (2.5 m moles), coenzyme A 11.4 mg (0.02 m mole), TMK solution (0.06 M KCl, 0.01 M magnesium acetate, 0.006 M 2-mercaptoethanol in tris buffer) in 10 times higher concentration 9 ml, enzyme solution from *E. coli* K 12 ML 1629 12 ml, total 30 ml. The determination of the activity by the cylinder plate method showed 64 mcg/ml of kanamycin. When it was heated in 2 N NaOH at 100°C for 8 hours, the activity only increased to 110 mcg/ml. The filtrate was lyophilized, and 4.654 g of yellowish powder was obtained. It was dissolved in 200 ml of distilled water (pH 6.6). The solution was passed through a column of Amberlite IRC-50 (100 ml of Na^+ form, pH 6.8) and after washing the column with 400 ml of distilled water, the inactivated kanamycin was eluted

with 1 % NH_4OH. The ninhydrin positive eluate was concentrated under reduced pressure and dried, yielding 210 mg of light brown powder. It was dissolved in 4 ml of distilled water and the solution was passed through a column of Amberlite CG-50 (25 ml of NH_4 form). After washing with water, the chromatograph was developed with 0.5 % NH_4OH. Ninhydrin-positive fractions were collected, concentrated under reduced pressure and dried, yielding 90 mg of white powder. It was dissolved in 2 ml of distilled water and passed through a column of Dowex 1 X 2 (25 ml of OH form) which was washed with water, and eluted with 0.5 N HCl. The ninhydrin-positive neutral eluate was concentrated and dried, yielding 47 mg of the inactivated kanamycin-I hydrochloride.

Another lot of the inactivated kanamycin was obtained by reaction (37°C, 24 hours) in the following solution: kanamycin sulfate 1.92 g (2.5 m moles), disodium ATP 3.8 g (18.8 m moles), $NaHCO_3$ 3.0 g (37.5 m moles), modified TMK solution (0.06 M KCl, 0.01 M $MgCl_2$, 0.006 M 2-mercaptoethanol in 0.1 M tris buffer) in 10 times higher concentration 48 ml, enzyme solution from *E. coli* K 12 ML 1629 72 ml, total 240 ml. The reaction mixture was heated at 100°C for 5 minutes and filtered, yielding 250 ml of filtrate (pH 5.8). The filtrate was passed through a column of Amberlite IRC-50 (200 ml of Na^+ form) and the colum was washed with water. The effluents were combined (1,500 ml, pH 5.8) and adjusted to pH 6.4 with 4 N NaOH and passed through another column of Amberlite IRC-50 (200 ml of Na^+ form) and the column was washed with 1,000 ml of distilled water. In the effluent, a slight amount of the inactivated kanamycin-II was shown by high-voltage paper electrophoresis. The inactivated kanamycin on the columns was eluted with 1 % NH_4OH and the ninhydrin positive eluate was concentrated under reduced pressure and dried. Then, 200 mg of a white powder was obtained from the eluate of the first column and 100 mg from the eluate of the second column. These powders were combined and dissolved in 10 ml of distilled water. The solution was passed through a column of Dowex 1 X 2 (25 ml of OH form) which was washed with 100 ml of distilled water and eluted with 0.5 N HCl. A ninhydrin-positive peak appeared after 37 ml of the eluate. This eluate was made pH 7.8 with NH_4OH and passed through a column of Amberlite CG-50 (25 ml of NH_4^+ form) and thereafter the column was washed with distilled water (100 ml). The ninhydrin-positive effluent (70 ml) containing the inactivated kanamycin-II was dried, yielding 60 mg of the inactivated kanamycin-II as a white powder. The column was eluted with 1 % NH_4OH and the ninhydrin-positive eluate (15 ml) was concentrated under reduced pressure and dried, yielding 90 mg of the inactivated kanamycin-I.

3. Isolation of the inactivated paromamine: Paromamine was inactivated at 37°C for 24 hours in the following reaction mixture: paromamine trihydrochloride 648 mg (1.5 m moles), disodium ATP 8,400 mg (14.0 m moles), $NaHCO_3$ 2,280 mg (27.2 m moles), modified TMK solution in 10 times higher concentration 120 ml, enzyme solution from *E. coli* K 12 ML 1629 60 ml, total 200 ml. The reaction mixture was heated at 100°C for 5 minutes and filtered. The filtrate was pH 6.0. It was passed through a column of Amberlite IRC-50 (400 ml of Na^+ form, pH 7.4) and after the column was washed with 1,000 ml of distilled water, eluted with 1 % NH_4OH. The positive ninhydrin eluate (100 ml) was concentrated under reduced pressure and dried, yielding 130 mg of a white powder. It was dissolved in 5 ml of water and passed through a column of Amberlite CG-50 (20 ml of NH_4^+ form, pH 9.4) and developed with distilled water. The ninhydrin positive effluent (45 ml) was concentrated and yielded 50 mg of white crystals of the inactivated paromamine by addition of methanol.

4. High-voltage paper electrophoresis of kanamycin, the inactivated kanamycins, paromamine and the inactivated paromamine: Toyo No. 51 paper (2×60 cm) and the high-voltage paper electrophoresis apparatus (Model HV 5,000-3) of Savant Instruments, Inc. were employed. The condition was as follows: acetic acid : formic acid : water (75 : 25 : 900 v/v), 3,500 V, 15 minutes. The positions were detected by ninhydrin reaction and

in the case of the ^{3}H-kanamycins also by radioactivity scanning. The distances of the movement toward the cathode were as follows: kanamycin 13.1~15.4 cm, the inactivated kanamycin-I 10.4~11.5 cm, the inactivated kanamycin-II 7.5~8.6 cm, paromamine 12.5~15.0 cm, the inactivated paromamine 7.8~9.8 cm. A compound in which the hydroxyl group of C-6 of 3-amino-3-dexoy-D-glucose moiety of kanamycin was phosphorylated was synthesized by S. UMEZAWA[3], and designated kanamycin-6'-phosphate. It moved 11.2 cm.

5. Thin-layer chromatography of kanamycin and the inactivated kanamycins: Silica gel G (Merck) was employed and two solvent systems were as follows: *n*-propanol: pyridine: acetic acid: water (51: 20: 6: 24, v/v) and *n*-butanol: acetic acid: water (4: 2: 1, v/v). In the former solvent, Rf values were as follows: kanamycin 0.29~0.44, the inactivated kanamycin-I 0.03, the inactivated kanamycin-II 0.03. In the latter solvent, all showed Rf 0.

6. Reactivation of the inactivated kanamycins and the inactivated paromamine: The inactivated kanamycin-I (1 mg/ml) was heated in 2 N NaOH at 100°C for 10 hours and then the pH was adjusted to 8.0 and the antibacterial activity was tested by a cylinder plate method, using *B. subtilis* as the test organism. The activity was 2.0 mcg/ml before the reaction and 3.4 mcg/ml after the reaction. The inactivated kanamycin-I (1 mg/ml) was heated in 0.5 N HCl at 100°C for 30 minutes, in 1 N HCl for 1 hour and 4 hours, and in 6 N HCl at 100°C for 30 minutes, and the recovery of the activity was 0.7%, 0.9%, 0.5%, <0.2% respectively. The inactivated kanamycin-I (0.4 mg/ml) was heated in 0.4 M $HClO_4$ at 80~83°C with or without adjustment to pH 4.0 or pH 6.8 for various times. The result is shown in Table 1. At pH 4.0, after 1, 3, 8, or 20 hours, the recovery of the activity was 3.0%, 6.3%, 16.3%, 80.0%. The reaction product for 20 hours was confirmed to be kanamycin by the high-voltage paper electrophoresis. The inactivated kanamycin-II was heated in 6 N HCl at 100°C for 30 minutes, and the recovery of the activity was less than 0.2%. It was heated in 0.4 M $HClO_4$ at pH 4.0 and 80~83°C for 20 hours. Then, 3.6% of the activity was recovered. The inactivated paromamine (0.4 mg/ml) was heated in 0.4 M HCl at pH 4.0 for 20 hours and the reactivated product was shown to be paromamine by high-voltage paper electrophoresis.

7. Periodate oxidation of kanamycin, inactivated kanamycins, paromamine and inactivated paromamine: The ultraviolet spectroscopic method reported by RAMMLER and RABINOWITZ[5] was employed and the optical density was read at 300 mμ in 1 M sodium acetate buffer of pH 4.05 at room temperature. The periodate consumption was as follows: 3.8 moles by kanamycin, 2.0 moles by inactivated kanamycin-I, 2.0 moles by inactivated kanamycin-II (molecular weight as 600), 4.4 moles by paromamine, 1.6 moles by inactivated paromamine after 24 hours.

8. Acid hydrolysis and thin-layer chromatography of inactivated kanamycin and inactivated paromamine: These products (5 mg/ml) were refluxed in 6 N HCl for 30 minutes and examined by the thin-layer chromatography. The chromatograms on Silica gel G (Merck) were developed by the following solvents: *n*-butanol: acetic acid: water (4: 2: 1, v/v) or *n*-propanol: pyridine: acetic acid: water (51: 20: 6: 24, v/v).

Inactivated kanamycin-I (5.6 mg, 9.9 μ moles) and II (6.2 mg) were oxidized by 25.2 μ moles of sodium periodate at room temperature for 38.5 hours. Then, 0.1 ml of ethyleneglycol was added and the product hydrolyzed with 6 N HCl under reflux for 30 minutes. The hydrolyzate was tested by the thin-layer chromatography with the results shown in Table 2.

9. Deamination followed by the acid hydrolysis of inactivated kanamycin-I: The starting material (11.2 mg) was dissolved in 0.5 ml of water, and 15 ml of 30% $NaNO_2$ and 3 ml of glacial acetic acid were added. The reaction was carried out at room temperature for 2 hours under stirring. The reaction solution was made alkaline with 4 N NaOH and passed through a column of active carbon. After washing with water, it

was eluted with 50 % methanol. The eluate was dried and hydrolyzed in 6 N HCl under reflux for 30 minutes and the hydrolyzate was tested by the thin-layer chromatography. No ninydrin positive spot was detected.

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