Inactivation of Kanamycin, Neomycin, and Streptomycin by Enzymes Obtained in Cells of *Pseudomonas aeruginosa*

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Ten strains of *Pseudomonas aeruginosa* were disrupted and centrifuged. The supernatant fluids from centrifugation at 105,000 × g contained enzymes inactivating kanamycin, neomycin, and streptomycin in the presence of adenosine triphosphate. Kanamycin-inactivating enzyme was precipitated with ammonium sulfate at 66% of saturated concentration, and the inactivated kanamycin was shown to be kanamycin-3'-phosphate in which the C-3 hydroxyl group of 6-amino-6-deoxy-D-glucose moiety was phosphorylated. This is identical with kanamycin inactivated by *Escherichia coli* carrying R factor. Streptomycin-inactivating enzyme was precipitated with ammonium sulfate at 33% of saturated concentration.

The resistance of *Escherichia coli* carrying R factor to chloramphenicol, streptomycin, and kanamycin has been known to be due to the formation of enzymes inactivating these antibiotics. As reported by Okamoto and Suzuki, a cell-free system of *E. coli* carrying R factor inactivates these antibiotics. Umezawa et al. (5) reported obtaining two kinds of inactivated kanamicins, depending on R factors introduced to *E. coli* K-12. In one, the amino group of 6-amino-6-deoxy-D-glucose moiety is acetylated (6) and in the other the C-3 hydroxyl group of the same moiety is phosphorylated (2, 5). Umezawa et al. (7) also reported that adenylylstreptomycin was formed by a cell-free system of *E. coli* carrying R factor in the presence of adenosine triphosphate (ATP).

Most strains of *Pseudomonas aeruginosa* are resistant to these antibiotics, but the mechanisms of the resistance have not been studied. It is suggested that the resistance is due to the formation of enzymes inactivating these antibiotics. We observed that the cell-free system obtained from *P. aeruginosa* contained enzymes inactivating kanamycin A, kanamycin B, kanamycin C, neomycin, neamine, paromomycin, streptomycin, dihydrostreptomycin, and chloramphenicol. In this paper, we report on studies on the inactivation of kanamycin, neomycin, and streptomycin by soluble enzymes obtained from *P. aeruginosa*, the purification of enzymes inactivating kanamycin and streptomycin, and the isolation and identification of the inactivated kanamycin.

**Materials and Methods**

*Strains of P. aeruginosa*. Ten strains (H1, H2, H3, H4, H5, H6, H7, H8, H9, and A1) were obtained from Y. Homma, Institute of Medical Science, University of Tokyo. The minimal concentrations of drug for inhibition of growth were as follows (in µg/ml): chloramphenicol, for all strains except H4, 160, and for H4, 40; tetracycline, for all strains, 40 to 80; streptomycin, for all strains, 40 to 160 or higher; neomycin, for H2, H3, H4, H7, and A1, 13 to 25, and for H1, H5, H6, H8, and H9, 50 to 100 or higher; kanamycin, for H2, H3, and H4, 40 to 80, and for H1, H5, H6, H7, H8, H9, and A1, 160 or higher; and gentamicin, for all strains except H4, 3 to 12, and for H4, less than 2.

*Antibiotics employed*. Crystalline kanamycin monosulfate, kanamycin B sulfate, kanamycin C sulfate, streptomycin sulfate, dihydrostreptomycin sulfate, and neamine were supplied by Meiji Seika Co., and neomycin sulfate by Nihon Kayaku Co. Gentamicin C sulfate was supplied by Shionogi Pharmaceutical Ind., Ltd.

*Procedures for preparation of S 105 fraction*. *P. aeruginosa* was grown in 0.3% glucose broth (0.3% glucose, 0.5% NaCl, 1.0% meat extract, 1.0% peptone, at pH 7.8) for 18 hr with shaking at 37 C. The culture thus obtained was diluted about 50-fold with the same medium and cultivated, with shaking, to the late logarithmic phase. The yield of cells was 3 to 4 g (wet weight) per liter. The cells were chilled rapidly by pouring the cultured broth onto crushed ice and were collected by centrifugation. The cells were washed twice with a buffer solution which contained 0.06 M KCl, 0.01 M magnesium acetate, 0.006 M 2-mercaptoethanol, and 0.1 M tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 7.8) and was designated.
TMK buffer solution. They were homogenized in an equal volume of the same buffer and disrupted by passage through a French pressure cell (400 kg/cm²). The homogenized material was digested by pancreatic deoxyribonuclease (4 µg/ml) and centrifuged at 10,000 × g for 30 min. The supernatant fluid was centrifuged at 105,000 × g for 2 hr. The supernatant fluid thus obtained was dialyzed against TMK buffer solution and was used as the enzyme solution which was designated S 105 fraction.

**Inactivation of antibiotics and assay method of the inactivating activity.** The reaction mixture contained S 105 fraction, antibiotics solution, ATP solution, KCl (final concentration, 156 mM), magnesium acetate (final concentration, 10 mM), 2-mercaptoethanol (final concentration, 15.6 mM), and Tris-chloride (final concentration, 260 mM), pH 7.5. After the inactivation, the reaction mixture was heated to 80°C for 5 min and centrifuged. The activity of the residual antibiotics in the supernatant fluid was determined by a disc plate method.

**Method of partial purification of kanamycin-inactivating enzyme.** To S 105 fraction prepared from *P. aeruginosa* H9 was added ammonium sulfate at 33% of the saturated concentration. The precipitate was discarded, and the enzyme in the supernatant fluid was precipitated by addition of ammonium sulfate at 66% of the saturated concentration. The precipitate was collected by centrifugation, dissolved in TMK buffer solution, and dialyzed against the same buffer. The 33 to 66% ammonium sulfate fraction (100 mg of protein) was loaded on a Sephadex G 50 column (14 × 360 mm) and was fractionated with a buffer which consisted of 0.06 M KCl, 0.01 M magnesium acetate, and 0.1 M Tris-buffer (pH 7.8). The effluent was cut into 3-ml fractions. The fractions showing the inactivation of kanamycin were combined and loaded on a Sephadex G 100 column (14 × 530 mm) and were fractionated by elution with the buffer solution described above. The effluent was cut into 3-ml fractions.

The kanamycin-inactivating activity of each fraction was determined as follows. The inactivation activity of an 0.1-ml amount of the fraction was tested in the reaction mixture described above (containing 2 mm kanamycin and 5 mM ATP) at 37°C for 1 hr, and the kanamycin inactivated was calculated in milligrams. This amount was multiplied by 10 (Fig. 5).

**Method of partial purification of streptomycin-inactivating enzyme.** Ammonium sulfate, at 33% saturation, was added to the S 105 fraction obtained from *P. aeruginosa* H9. The precipitate was collected, dissolved in the TMK solution, and dialyzed against the same buffer. The dialyzed solution (150 mg of protein) was loaded on a Sephadex G 100 column (14 × 530 mm), and chromatography was developed as described above.

The streptomycin-inactivating activity of each fraction was determined by the same procedure for the kanamycin-inactivating activity, but, in this case, streptomycin was added at 0.2 mM to the reaction mixture and the reaction was carried at 37°C for 3 hr.

**RESULTS AND DISCUSSION**

**Inactivation reactions and partial purification of the inactivating enzymes.** Cell-free systems of all 10 strains of *P. aeruginosa* contained enzymes inactivating kanamycin, neomycin, and streptomycin in the presence of ATP. In our experiments (Table 1), the inactivation reaction was carried in the reaction mixture consisting of 0.2 mM or 1 mM of these antibiotics, 20 mM ATP, and 3 mg (as protein)/ml of S 105 fractions of 10 strains of *P. aeruginosa*, 156 mM KCl, 10 mM magnesium acetate, 15.6 mM 2-mercaptoethanol, 260 mM Tris-buffer (pH 7.5) at 37°C for 20 hr. The residual active antibiotics were determined by disc methods, and the inhibition percentages were calculated. The enzymes inactivating these antibiotics must be in cells, because no inactivation was observed when these antibiotics were shaken with the intact cells.

The time course of the inactivation was studied on S 105 fraction obtained from the strain H 9. The concentrations of ATP and the S 105 fraction in the reaction mixture were 20 mM and 0.3 mg (protein)/ml, respectively. Kanamycin and neomycin were added at 1 mM and streptomycin was added at 0.2 mM concentration. The reaction was made at 37°C (Fig. 1). With kanamycin and neomycin, the rate of inactivation was high during the first 2 hr and, in the case of streptomycin, it was high during the first 1 hr. As described later, kanamycin and streptomycin are inactivated by different enzymes. The time courses of inactivation of kanamycin and neomycin are similar, and it is suggested that they are inactivated by the same enzyme.

ATP is necessary for the inactivation of the antibiotics. If ATP is omitted, then no inactivation occurs (Fig. 2). In the reaction mixture in the absence of ATP, the inactivation of kanamycin, neomycin, and streptomycin was insignificant.

**Table 1. Inactivation of kanamycin, neomycin, and streptomycin by S 105 fractions of Pseudomonas aeruginosa**

<table>
<thead>
<tr>
<th>Pseudomonas aeruginosa strain</th>
<th>Inactivation (%)</th>
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<tbody>
<tr>
<td></td>
<td>Kanamycin</td>
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<tr>
<td>0.2 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>H1</td>
<td>66</td>
</tr>
<tr>
<td>H2</td>
<td>76</td>
</tr>
<tr>
<td>H3</td>
<td>42</td>
</tr>
<tr>
<td>H4</td>
<td>37</td>
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<td>H5</td>
<td>47</td>
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<td>H6</td>
<td>100</td>
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<tr>
<td>H7</td>
<td>71</td>
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<tr>
<td>H8</td>
<td>100</td>
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<tr>
<td>H9</td>
<td>100</td>
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<td>As</td>
<td>50</td>
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experiment shown in Fig. 2, concentrations of S 105 fraction and the antibiotics were 1.8 mg (as protein)/ml, and 5 mM kanamycin, 5 mM neomycin, or 0.2 mM streptomycin. Concentration of ATP varied, and the reaction was carried for 20 hr at 37 C. The increase of ATP up to about 20 mM raised the inactivation percentage of these antibiotics. If the concentration of ATP exceeded 20 mM, then a slight decrease in the percentage of inactivation was observed, especially in the case of kanamycin. Addition of cytidine triphosphate (CTP), guanosine triphosphate (GTP), or uridine triphosphate (UTP), instead of ATP, was observed to cause the inactivation reaction. When ATP, CTP, GTP, or UTP was added at 2 mM to the reaction mixture contain-
tested without addition of magnesium acetate and with addition of 0.1 mM ethylenediaminetetraacetate. Then, inactivation as low as 40% was observed. Whether the inactivation reactions by the purified enzymes require a metal ion is of interest.

The optimal pH for an inactivation reaction of kanamycin is about 7.5 and that for streptomycin is about 8.5. Kanamycin at 0.2 mM or streptomycin at 0.2 mM was inactivated in the presence of 20 mM ATP and 0.2 mg (as protein)/ml of S 105 fraction (obtained from strain H9) at 37 C for 30 min and at various pH values (Fig. 4). The optimal pH for inactivation of kanamycin is the same as that for phosphorylative inactivation of kanamycin by S 105 of E. coli ML 1629 carrying R factor, and the optimal pH for streptomycin is the same as that for streptomycin inactivation by S 105 of the same strain of E. coli which catalyzed the reaction from streptomycin and ATP to adenylyl streptomycin.

The inactivating enzyme of kanamycin in the S 105 fraction of strain H9 was partially purified. The enzyme is partially precipitated by 33% saturation ammonium sulfate but completely precipitated by 66% saturation. Therefore, the precipitate obtained by 33 to 66% saturation of ammonium sulfate was loaded on a Sephadex G 50 column, and the buffer solution consisting of 0.06 M KCl, 0.01 M magnesium acetate, 0.1 M Tris-chloride (pH 7.8) was used as eluant. The fractions showing the inactivation activity were collected and further purification was made by column chromatography on Sephadex G 100. The activity in the effluent is shown in Fig. 5. The result indicates that this process is useful for purification of the kanamycin-inactivating enzyme. The enzyme inactivating streptomycin is precipitated by 33% saturation of ammonium sulfate. This fraction was loaded on a Sephadex G 100 column and the buffer solution was used as eluant. The activities inactivating streptomycin and kanamycin are shown in Fig. 6. Comparing the result in Fig. 6 with that in Fig. 5, the kanamycin-inactivating activity appeared in the same tube numbers of the effluent in both experiments. This result indicates that the kanamycin-inactivating enzyme is partially precipitated by 33% saturation of ammonium sulfate. The elution pattern shown in Fig. 6 and the precipitation of streptomycin-inactivating enzyme by 33% saturation of ammonium sulfate indicate that streptomycin is inactivated by a different enzyme from that for kanamycin.

Isolation and identification of inactivated kanamycin. Kanamycin was inactivated in a reaction mixture consisting of 10 mM kanamycin, 20 mM ATP, 4 mg (as protein)/ml of S 105 fraction of strain H9, and other materials (shown in Materials and Methods). From the 128 ml of the reaction mixture, the inactivated kanamycin was extracted and purified by the ion-exchange resin chromatography described by Kondo et al. (2) for isolation of the phosphorylated kanamycin. The yield calculated from the reaction mixture to the purified inactivated kanamycin was 59%. The inactivated kanamycin darkened at 250 to 260 C. The formula calculated by the elemental analysis was as follows. Analysis: C_{18}H_{33}N_{4}O_{7}; PO(OH)_{2}.2H_{2}O; calculated: C, 36.00; H, 6.88; N, 9.33; O, 42.63; P, 5.16; found: C, 34.93; H-7.06; N, 8.99; O, 40.19; P, 5.20. Four amino

![Fig. 4. Effects of pH on the inactivation of kanamycin and streptomycin by S 105 fraction of Pseudomonas aeruginosa H9.](http://aem.asm.org/Downloaded-from)
groups were shown by the Van Slyke method and the inactivated kanamycin was converted to kanamycin by heating at 80°C for 6 hr in 0.4 M perchloric acid at pH 4.0 and also by treatment with alkaline phosphatase. It consumed 2 moles of periodate, and 6-amino-6-deoxy-D-glucose was shown by high voltage electrophoresis after periodate oxidation and acid hydrolysis. Upon thin-layer chromatography, high-voltage electrophoresis and paper chromatography, and infrared spectrum, results were the same as those of the inactivated kanamycin I (kanamycin-3′-phosphate) described by Kondo et al. (2), in which C-3 hydroxyl group of 6-amino-6-deoxy-D-glucose moiety is phosphorylated.

Therefore, it is concluded that the inactivated kanamycin obtained by the inactivation with an enzyme of *P. aeruginosa* is the same as that obtained by phosphorylative inactivation with an enzyme of *E. coli* ML 1629 carrying R factor. As reported in another paper (1), an enzyme solution prepared from drug-resistant staphylococci phosphorylates and inactivates kanamycin, and the inactivated kanamycin has the same structure.

As described by Okanishi et al. (4), *E. coli* ML 1629 carrying R factor is resistant to kanamycin, kanamycin B, kanamycin C, paromomycin, neomycin, and neamine, and S 105 fraction of this *E. coli* phosphorylates C-3 hydroxyl group of 6-amino-6-deoxy-D-glucose of kanamycin and C-3 hydroxyl of glucosamine of paromomycin; phosphorylation of the corresponding hydroxyl groups in neamine, neomycin, and paromomycin is suggested. S 105 fraction of *P. aeruginosa* H9 strain was proved to inactivate not only kanamycin and neomycin but also kanamycin B, kanamycin C, paromomycin, and neamine under the same conditions as for inactivation of kanamycin. Therefore, it is considered that C-3 hydroxyl group of D-2,6-diamino-2,6-dideoxy-D-glucose of D-glucosamine

![Graph](http://aem.asm.org/)
moieties of these antibiotics is phosphorylated by S 105 of \textit{P. aeruginosa}.

\textbf{Literature Cited}


\textbf{FIG. 6.} Elution profile of Sephadex G 100 column chromatography of the precipitate of S 105 fraction of \textit{Pseudomonas aeruginosa} H9 at 33\% saturation of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Elution profile of Sephadex G 100 column chromatography of the precipitate of S 105 fraction of \textit{Pseudomonas aeruginosa} H9 at 33\% saturation of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}.}
\end{figure}