PHOSPHORYLATION AND INACTIVATION OF KANAMYCIN BY *PSEUDOMONAS AERUGINOSA*

Sir:

As reported by UMEZAWA *et al.*[1,2], enzymes acetylating amino group of 6-amino-6-deoxy-D-glucose moiety of kanamycin, phosphorylating C-3 hydroxyl group of the same moiety of kanamycin, or phosphorylating C-3 hydroxyl group of D-glucosamine moiety of paromamine are formed in cells of *E. coli* carrying R factor. These acetylated or phosphorylated products show no effect on polyphenylalanine synthesis by the ribosome system of *E. coli* with polyuridylic acid, and do not cause misreading of the codes on mRNA except for a slight effect of the acetylated product[3]. On the basis of the absence of such enzymes in a sensitive strain and the occurrence of cross resistance to related antibiotics, resistance of *E. coli* carrying R factor to kanamycin can be explained by the presence of enzymes inactivating this antibiotic. It has been also reported by UMEZAWA *et al.*[4] that *E. coli* carrying R factor forms an enzyme catalyzing the formation from streptomycin and ATP or ADP of adenylyl-streptomycin.

Most strains of *Pseudomonas aeruginosa* are resistant to various antibiotics. The natural resistance is now suspected to be related to the formation of enzymes which inactivate antibiotics. The authors have observed enzymes inactivating kanamycin and streptomycin in cells of *Pseudomonas*. In this paper, inactivation of kanamycin by an enzyme solution obtained from *Pseudomonas aeruginosa* and isolation of the inactivated kanamycin, which is identical with that phosphorylated by an enzyme solution prepared from *E. coli* carrying R factor, are reported.

Pseudomonas aeruginosa strain H-9 was grown in 0.3% glucose broth at 37°C. The cells were harvested in the late logarithmic phase of growth, and disrupted by passage through a French pressure cell (400 kg/cm^2). The extract was digested by pancreatic DNase and repeatedly centrifuged. The 105,000×*g* supernatant was dialyzed and used as the enzyme. Kanamycin was inactivated at 30°C for 20 hours in the following reaction medium: the 105,000×*g* supernatant of *Pseudomonas aeruginosa* 4 mg protein/ml, kanamycin sulfate 10 mM, ATP 20 mM, KCl 156 mM, Mg acetate 20 mM, 2-mercaptoethanol 15.6 mM and Tris 260 mM, pH 8.2.

After the reaction, the reaction mixture in which 620 mg of kanamycin was inactivated was centrifuged, and the supernatant (128 ml) was passed through a column (150 ml) of Amberlite IRC-50 resin (Na^+ form). After washing with 500 ml of distilled water, the column was eluted with 1% NH_4OH (100 ml). The ninhydrin positive fraction was collected and dried *in vacuo*. The crude powder (867 mg) thus obtained was dissolved in water and subjected to column chromatography on Dowex 1×8 resin (70 ml of NH_4^+ form) developed with water (300 ml) and 0.5 N HCl (250 ml). The ninhydrin-positive fraction eluted with 0.5 N HCl was concentrated *in vacuo* and dried, yelding 414 mg of a light brown powder. It was further purified by column chromatography on Amberlite CG-50 resin (50 ml of NH_4^+ form). A ninhydrin-positive fraction appeared with 1% NH_4OH and after concentration and drying yielded 347 mg of the inactivated kanamycin which showed one spot (Rf 0.06) on thin-layer chromatography using propanol – pyridine – acetic acid – water (51 : 20 : 6 : 24, v/v) and also on high voltage paper electrophoresis.

The structure of the inactivated product of kanamycin was determined, following the procedure described by KONDO *et al.*[5] It has the following properties: darkening at 250~260°C; but not 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 34.93, H 7.06, O 40.19, N 8.99, P 5.20; positive ninhydrin and positive test for phosphorous by HANES reagent. The inactivated product is a monophosphorylated kanamycin as demonstrated by the analytical result and by the behavior on paper electrophoresis. It moved toward the cathode by 16.1~16.4 cm on the high voltage paper electrophoresis, using acetic acid –

formic acid – water (75 : 25 : 900, v/v), 3,500 V for 15 minutes. Kanamycin moved toward the cathode 20.5 cm. The inactivated product was converted to kanamycin by heating in 0.4 M $HClO_4$ adjusted to pH 4.0 at 80~83°C or by hydrolysis with alkaline phosphatase and the antimicrobial activity was recovered. The inactivated kanamycin consumed 2.0 moles of periodate at pH 4.05 in 24 hours, while kanamycin consumed 4.4 moles. Four amino nitrogens were shown by VAN SLYKE method both in kanamycin and the inactivated product. It showed no maximum in the ultraviolet spectrum.

The identity of the inactivated kanamycin with the inactivated kanamycin-I described by KONDO *et al.*[5] in which C-3 hydroxyl group of 6-amino-6-deoxy-D-glucose moiety is phosphorylated, and which is obtained by treatment of kanamycin with an enzyme solution prepared from *E. coli* ML 1629 carrying R factor, is shown by similar behavior on high voltage paper electrophoresis, on thin-layer chromatography using propanol – pyridine–acetic acid – water (51 : 20 : 6 : 24, v/v) and by presence of 6-amino-6-deoxy-D-glucose in the hydrolysate of periodate-oxydized material.

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References

1) UMEZAWA, H.; M. OKANISHI, R. UTAHARA, K. MAEDA & S. KONDO: Isolation and structure of kanamycin inactivated by a cell free system of kanamycin-resistant *E. coli*. J. Antibiotics, Ser. A 20 : 136~141, 1967

2) UMEZAWA, H.; M. OKANISHI, S. KONDO, K. HAMANA, R. UTAHARA, K. MAEDA & S. MITSUHASHI: Phosphorylative inactivation of aminoglycosidic antibiotics by *Escherichia coli* carrying R factor. Science 157 : 1559~1561, 1967

3) HORI, M. & H. UMEZAWA: Miscoding activities of biologically inactivated kanamycins. J. Antibiotics, Ser. A 20 : 386~387, 1967

4) UMEZAWA, H.; S. TAKAZAWA, M. OKANISHI & R. UTAHARA: Adenylylstreptomycin, an inactivated product of streptomycin by *E. coli* carrying R factor. J. Antibiotics, 21 : 81~82, 1968

5) KONDO, S.; M. OKANISHI, R. UTAHARA, K. MAEDA & H. UMEZAWA: Isolation of kanamycin and paromamine inactivated by *E. coli* carrying R factor. J. Antibiotics 21 : 22~29, 1968