ADENYLYLDIDEOXYKANAMYCIN B, A PRODUCT OF THE INACTIVATION OF DIDEOXYKANAMYCIN B BY *ESCHERICHIA COLI* CARRYING R FACTOR

Sir:

In previous papers[1~11], it was reported that acetylation, phosphorylation and adenylylation were the mechanisms of inactivation of aminoglycosidic antibiotics by *Escherichia coli* carrying R factor and *Pseudomonas aeruginosa*. It was found that kanamycin was inactivated by phosphorylation of the 3-hydroxyl group of 6-amino-6-deoxy-D-glucose moiety by *E. coli* carrying R factor, strain ML-1629[3~5], and *P. aeruginosa*[7,10,11]. Recently, UMEZAWA *et al.*[12] synthesized 3′,4′-dideoxykanamycin B (DKB) which was effective against kanamycin-resistant *E. coli* ML-1629 and *P. aeruginosa* strains. However, DKB at a concentration of 50 μg/ml did not inhibit *E. coli* JR66/W677 which had been studied by BENVENISTE and DAVIES[13]. They reported that this strain was obtained by transmission of R factor to sensitive strain *E. coli* W677, from *Klebsiella pneumoniae* strain type 22 # 3038 which was clinically isolated and was resistant to kanamycin, streptomycin and seven other drugs. *E. coli* JR66/W677 was resistant to kanamycin A (>100 μg/ml), kanamycin B (>100 μg/ml), streptomycin (50 μg/ml) and gentamicin (25 μg/ml).

In this paper, inactivation of DKB by an enzyme preparation from *E. coli* JR66/W677, and isolation and characterization of the inactivated product are presented.

The enzyme solution was prepared from logarithmically growing cells in the nutrient broth containing 5 μg/ml of DKB. The method of OKANISHI *et al.*[4] was modified as follows; growing cells of *E. coli* JR66/W677 were washed twice with 20 mM potassium phosphate buffer (pH 7.8) containing 10 mM magnesium acetate and 6 mM 2-mercaptoethanol, and suspended in an equal volume of the same buffer; the cell suspension was passed through a French pressure cell (1,200 kg/cm^2) and then centrifuged at 100,000 *g* for 90 minutes at 4°C; protein content of the supernatant was determined by the FOLIN method, and an enzyme solution containing 10 mg/ml of protein was prepared by dilution with the buffer described above. The optimum pH of the enzymatic reaction was 7.5~8.0. In an experiment, 90 mg (0.2 mmoles) of DKB was inactivated in 500 ml of the reaction mixture containing 200 ml of the enzyme solution (2,000 mg of protein), 9,090 mg (15.1 mmoles) of disodium ATP, 800 mg (9.5 mmoles) of $NaHCO_3$, 8,012 mg (46 mmoles) of K_2HPO_4, 544 mg (4 mmoles) of KH_2PO_4, 1,072 mg (5 mmoles) of $Mg(CH_3COO)_2 \cdot 4H_2O$ and 232.3 mg (3 mmoles) of 2-mercaptoethanol. After 15-hour incubation at 37°C, 100 % inactivation of DKB was confirmed by the assay of the remaining activity. The disc-plate method using *Bacillus subtilis* PCI 219 as a test organism was employed. After reaction, the mixture was diluted to 1,000 ml with distilled water and kept in a boiling water bath for 10 minutes. After cooling, it was filtered and the filtrate was passed through a column of Amberlite CG-50 (NH_4^+ form, 100 ml). The column was washed with 2,000 ml of water and the inactivated DKB (IDKB) on the column was eluted with 0.2 % ammonia. The eluate with positive ninhydrin reaction and UV absorption at 254 mμ was concentrated to dryness, yielding 115 mg of a yellowish powder. The powder was rechromatographed on Amberlite CG-50, yielding 88.0 mg of slightly yellowish powder. Eighty mg of the powder was dissolved in 80 ml of 0.1 M ammonium formate and adsorbed on a column of CM-Sephadex C-25 (40 ml) equillibrated with 0.1 M ammonium formate. The column was eluted stepwise with 0.1 M, 0.2 M, 0.4 M, 0.6 M and 0.8 M ammonium formate, recording the UV absorption at 260 mμ. The fractions eluted at 0.8 M showed UV absorption. These fractions were collected and diluted with water to 2,000 ml. The diluted solution was passed through a column of Amberlite CG-50 (NH_4^+ form, 20 ml) and IDKB was eluted with 0.2 % ammonia. Thus, 66 mg of purified IDKB was obtained as a white powder.

IDKB darkens at 205~209°C but does not

melt at 280°C. Anal. calcd. for $C_{28}H_{49}N_{10}O_{14}P \cdot 3H_2O$: C 40.25, H 6.64, N 16.77, P 3.71. Found: C 40.40, H 6.76, N 16.42, P 3.96. It gives positive ninhydrin, RYDON-SMITH[14] and HANES[15] reactions. On high-voltage paper electrophoresis at 3,500 volts for 15 minutes, using acetic acid - formic acid - water (75 : 25 : 900), IDKB moves to the cathode 13.4 cm, while DKB moves 16.8 cm. IDKB shows a UV maximum at 260 mμ (ε=15,400) in water, and a maximum at 258 mμ (ε=14,400) in 0.1 N hydrochloric acid. IDKB is hydrolyzed to DKB and adenylic acid by snake venom phosphodiesterase purchased from Boehringer Mannheim Co. Thus, IDBK is a monoadenylyl DKB. As reported in another paper[16] the structure of IDKB was determined by n.m.r. analysis to be 3′,4′-dideoxykanamycin B-2′′-adenylate, that is, the 2-hydroxyl group of the 3-amino-3-deoxy-D-glucose moiety is adenylylated.

MORIMASA YAGISAWA
HIROSHI NAGANAWA
SHINICHI KONDO
MASA HAMADA
TOMIO TAKEUCHI
HAMAO UMEZAWA

Institute of Microbial Chemistry,
Shinagawa-ku, Tokyo, Japan

(Received October 25, 1971)

References

1) OKANISHI, M.; S. KONDO, Y. SUZUKI, S. OKAMOTO & H. UMEZAWA: Studies on inactivation of kanamycin and resistance of *E. coli*. J. Antibiotics, Ser. A 20 : 132～135, 1967
2) 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
3) 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
4) OKANISHI, M.; S. KONDO, R. UTAHARA & H. UMEZAWA: Phosphorylation and inactivation of aminoglycosidic antibiotics by *E. coli* carrying R factor. J. Antibiotics 21 : 13～21, 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
6) UMEZAWA, H.; S. TAKASAWA, M. OKANISHI & R. UTAHARA: Adenylylstreptomycin, a product of streptomycin inactivated by *E. coli* carrying R factor. J. Antibiotics 21 : 81～82, 1968
7) UMEZAWA, H.; O. DOI, M. OGURA, S. KONDO & N. TANAKA: Phosphorylation and inactivation of kanamycin by *Pseudomonas aeruginosa*. J. Antibiotics 21 : 154～155, 1968
8) MAEDA, K.; S. KONDO, M. OKANISHI, R. UTAHARA & H. UMEZAWA: Isolation of paromamine inactivated by *Pseudomonas aeruginosa*. J. Antibiotics 21 : 458～459, 1968
9) TAKASAWA, S.; R. UTAHARA, M. OKANISHI, K. MAEDA & H. UMEZAWA: Studies on adenylylstreptomycin, a product of streptomycin inactivation by *E. coli* carrying the R factor. J. Antibiotics 21 : 477～484, 1968
10) DOI, O.; M. OGURA, N. TANAKA & H. UMEZAWA: Inactivation of kanamycin, neomycin, and streptomycin by enzymes obtained in cells of *Pseudomonas aeruginosa*. Appl. Microbiol. 16 : 1276～1281, 1968
11) DOI, O.; S. KONDO, N. TANAKA & H. UMEZAWA: Purification and properties of kanamycin-phosphorylating enzyme from *Pseudomonas aeruginosa*. J. Antibiotics 22 : 273～282, 1969
12) UMEZAWA, H.; S. UMEZAWA, T. TSUCHIYA & Y. OKAZAKI: 3′, 4′-Dideoxykanamycin B active against kanamycin-resistant *Escherichia coli* and *Pseudomonas aeruginosa*. J. Antibiotics 24 : 485～487, 1971
13) BENVENISTE, R. & J. DAVIES: R-Factor mediated gentamicin resistance: a new enzyme which modifies aminoglycoside antibiotics. FEBS Letters 14 : 293～296, 1971
14) RYDON, H. N. & P.W.G. SMITH: A new method for the detection of peptides and similar compounds on paper chromatograms. Nature 169 : 922～923, 1952
15) HANES, C. S. & F. A. ISHERWOOD: Separation of the phosphoric esters on the esters on the filter paper chromatogram. Nature 164 : 1107～1112, 1949
16) NAGANAWA, H.; M. YAGISAWA, S. KONDO, T. TAKEUCHI & H. UMEZAWA: The structure determination of an enzymatic inactivation product of 3′, 4′-dideoxykanamycin B. J. Antibiotics 24 : 913～914, 1971