INACTIVATION OF 3′,4′-DIDEOXY-KANAMYCIN B BY AN ENZYME SOLUTION OF RESISTANT *E. COLI* AND ISOLATION OF 3′,4′-DIDEOXYKANAMYCIN B 2″-GUANYLATE AND 2″-INOSINATE

Sir:

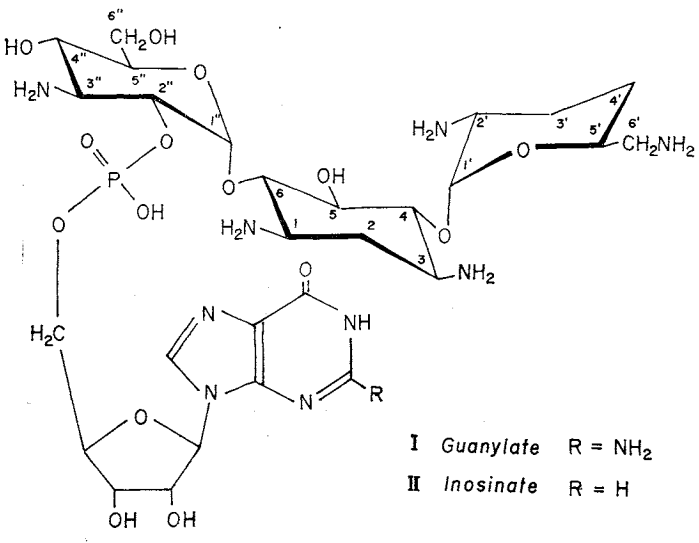
In previous papers[1,2] we reported inactivation of 3′,4′-dideoxykanamycin B (DKB) by a 105,000 g supernatant of disrupted cells of *Escherichia coli* JR66/W677 carrying R factor and isolation of the inactivated product, 3′,4′-dideoxykanamycin B 2″-adenylate. In this communication, we report inactivation of DKB by the same enzyme solution in the presence of various nucleoside triphosphates and isolation of the inactivated products, 3′, 4′-dideoxykanamycin B 2″-guanylate (I) and 2″-inosinate (II).

Inactivation of DKB was found to occur at 37°C under the following conditions. The reaction mixture consisted of 0.2 μmoles DKB, 0.1 ml of enzyme solution (10 mg protein/ml) prepared by the procedure described in a previous paper[1], 0.1 ml of 0.1 M $NaHCO_3$ containing 16 μmoles of a nucleoside triphosphate shown in Table 1, 0.1 ml of 1 M KH_2PO_4-K_2HPO_4 buffer (pH 7.8), 0.1 ml of 0.1 M $Mg(CH_3COO)_2 \cdot 4H_2O$, 0.1 ml of 0.06 M 2-mercaptoethanol and 0.5 ml of distilled water. After 1, 3 and 24 hours, 0.1 ml of the reaction mixture was diluted with 0.3 ml of 0.1 M KH_2PO_4-K_2HPO_4 buffer (pH 7.8) and kept in a boiling water bath for 10 minutes; the residual DKB was measured by a disc-plate method using *Bacillus subtilis* PCI 219.

As shown in Table 1, DKB was inactivated in the reaction mixture containing GTP, CTP, UTP or ITP as well as in that containing ATP. Less inactivation was observed in the reaction mixture containing deoxyribonucleoside triphosphates.

In order to determine if the nucleoside triphosphates actually react with DKB in this enzymatic inactivation reaction, we isolated the products from the reaction mixture containing GTP or ITP.

DKB was inactivated in the following reaction mixture: 1.8 mg DKB (4 μmoles), 2 ml of the enzyme solution (20 mg protein), 2 ml of 0.1 M $NaHCO_3$ containing 189.5 mg trisodium GTP (320 μmoles), 2 ml of 1 M KH_2PO_4-K_2HPO_4 buffer (pH 7.8), 2 ml of 0.1 M $Mg(CH_3COO)_2 \cdot 4H_2O$, 2 ml of 0.06 M 2-mercaptoethanol and distilled water to 20 ml. The reaction was carried out at 37°C for 20 hours. After reaction, the mixture was diluted with distilled water to 40 ml and kept in a boiling water bath for 10 minutes. The solution thus obtained was passed through a column of Amberlite CG-50 (NH_4^+ form, 5 ml), and the inactivated DKB (GDKB) was eluted with 0.1 N NH_4OH ammonia, yielding a yellow powder (6.4 mg)



I *Guanylate* R = NH_2

II *Inosinate* R = H

Table 1. Effect of nucleoside triphosphates on the inactivation of DKB by 105,000 g supernatant of disrupted cells of *E. coli* JR66/W677

Nucleotide	Inactivation %*		
	1 hour	3 hours	24 hours
ATP	36	85	100
GTP	12	40	100
CTP	39	63	100
UTP	22	54	100
ITP	4	85	100
dATP	42	50	85
dGTP	34	36	80
dCTP	20	34	84
dTTP	4	9	68
none	0	4	0

* Inactivation % were calculated from the residual DKB activity determined by the disc-plate assay using *Bacillus subtilis* PCI 219 as test organism.

By rechromatography of the powder on a column of Amberlite CG-50 (NH_4^+ form, 5 ml), 2.0 mg of a yellowish powder was obtained.

GDKB showed UV absorption spectrum similar to guanylic acid. On high-voltage paper electrophoresis with 3,500 volts for 15 minutes using acetic acid - formic acid - water (75 : 25 : 900), GDKB moved toward the cathode 9.5 cm, while DKB moved 14.0 cm and DKB 2''-adenylate 10.7 cm. GDKB showed no antibacterial activity. Snake venom phosphodiesterase hydrolyzed GDKB to DKB and guanylic acid which were detected by UV absorption, ninhydrin reaction on high-voltage paper electrophoresis and antibacterial activity. As reported[1], the inactivated DKB which was obtained after reaction with ATP was 3',4'-dideoxykanamycin B 2''-adenylate and therefore it is likely that the inactivated DKB obtained is 3',4'-dideoxykanamycin B 2''-guanylate.

DKB was inactivated at 37°C for 18 hours in the reaction mixture containing ITP as follows: 36 mg DKB (80 μmoles), 40 ml of enzyme solution (400 mg protein), 20 ml of 0.1 M $NaHCO_3$ containing 3,674.8 mg (6.4 mmoles) of trisodium ITP, 20 ml of 1 M KH_2PO_4-K_2HPO_4 buffer (pH 7.8), 20 ml of 0.1 M $Mg(CH_3COO)_2 \cdot 4H_2O$, 20 ml of 0.06 M 2-mercaptoethanol and distilled water to 200 ml. After 3-hour incubation, 56 % of DKB was inactivated and 100 % after 18 hours. After inactivation, the mixture was diluted with 200 ml of distilled water and kept in a boiling water bath for 10 minutes. The solution was filtered and the filtrate was passed through a column of Amberlite CG-50 (NH_4^+ form, 20 ml), the column was washed with 2,000 ml of distilled water and the inactivated DKB was eluted with 0.1 N NH_4OH. The eluate with UV absorption at 254 nm and positive nynhydrin reaction was concentrated to dryness, yielding 63.8 mg of yellow powder. The powder was rechromatographed on Amberlite CG-50, yielding 56.4 mg of yellowish powder. In chromatography on CM-Sephadex C-25 equillibrated with 0.1 M ammonium formate, inactivated DKB was eluted with 0.6 M ammonium formate. After chromatography on Amberlite CG-50 to remove ammonium formate, 47.7 mg of purified inactivated DKB (IDKB) was obtained as a white powder.

IDKB darkened at 232~239°C. Anal. calcd. for $C_{28}H_{48}N_9O_{15}P \cdot 4H_2O$: C 39.39, H 6.61, N 14.77, P 3.63. Found: C 39.15, H 6.47, N 14.90, P 3.55. It gave positive ninhydrin, Rydon-Smith[3] and Hanes[4] reactions. On high-voltage paper electrophoresis with 3,500 volts for 15 minutes, using acetic acid - formic acid - water (75 : 25 : 900), IDKB moved toward the cathode 8.2 cm, while DKB moved 11.4 cm and DKB 2''-adenylate 8.8 cm. It showed UV maxima at 250 nm (ε 11.5×10^3) in neutral aqueous solution and 0.1 N hydrochloric acid, and 254 nm (ε 12.3×10^3) in 0.1 N sodium hydroxide. It showed no inhibition zone by the disc-plate assay method using *B. subtilis* PCI 219 at a concentration of 1 mg/ml. IDKB was hydrolyzed to DKB and inosinic acid by snake venom phosphodiesterase.

The pmr spectrum of IDKB in D_2O solution (20 mg/0.3 ml, pH 8.8) using tetramethylsilane as an external reference ($\delta = 0$) showed signals of base ring protons of inosinyl moiety at $\delta = 8.65$ (1H) and 8.81 (1H), and protons of the ribose moiety at $\delta = 6.54$ (1H), 5.32 (1H), 4.99 (1H), 4.84 (1H) and 4.65 (2H). From the results of decoupling experiments, the signals at $\delta = 5.45$, 4.37 and 3.52 ppm could be assigned to 1''-H, 2''-H and 3''-H signals. Thus, the 2''-H singnal in IDKB shifted by 0.39 ppm to the lower-field compared with that of DKB. A similar shift was observed in the case of DKB 2''-adenylate[2]. Irradiation of the 1''-H and 3''-H signals caused the reduction of the multiplet of 2''-H at δ 4.37 to a doublet ($J_{2'',P} = 8.5$ Hz). Irradiation of the 1''-H and ^{31}P (40.490158 MHz) signals collapsed the 2''-H signal to a doublet ($J_{2'',3''} = 10.0$ Hz). Thus the structure of IDKB was determined to be 3',4'-dideoxykanamycin B 2''-inosinate.

Isolation of 3',4'-dideoxykanamycin B 2''-guanylate and 2''-inosinate suggests that an enzyme in the 105,000 g supernatant can catalyze the reaction of DKB with various nucleoside triphosphates.

Morimasa Yagisawa
Hiroshi Naganawa
Shinichi Kondo

TOMIO TAKEUCHI
HAMAO UMEZAWA

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

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