GENTAMICIN ACETYLTRANSFERASE IN *ESCHERICHIA COLI* CARRYING R FACTOR

Sir:

As reported by WITCHITZ,[1] *Escherichia coli* K12 C600 R135 is resistant to gentamicins but not kanamycin, tobramycin (3′-deoxykanamycin B), paromomycin and lividomycin. One of the authors, CHABBERT, was interested in the mechanism of its resistance and we confirmed that this resistance was dependent on gentamicin acetyltransferase: gentamicins C_{1a}, C_1 and C_2 were inactivated, but kanamycin and 3′, 4′-dideoxykanamycin B did not undergo this enzymic reaction. In this communication, we report the acetylation of the 3-amino group of the 2-deoxystreptamine moiety of gentamicin C_1 by this enzyme.

The enzyme solution was prepared from logarithmically growing cells of the strain in a nutrient broth containing 10 μg/ml of gentamicin C sulfate. The cells were harvested by centrifugation and washed twice with 20 mM potassium phosphate buffer (pH 7.2) containing 10 mM magnesium acetate, 60 mM potassium chloride and 10 mM 2-mercaptoethanol. The washed cells were suspended in an equal volume of the buffer described above and disrupted by passage through a French pressure cell (1,200 kg/cm²). The ruptured cell suspension was centrifuged at 100,000 *g* for 90 minutes, and the supernatant was diluted with the buffer to 10 mg/ml protein determined by LOWRY's method. The diluted enzyme solution was designated S-100.

The inactivation of gentamicin C_1 (95.5 mg, 200 μmoles) was carried out in the reaction mixture (200 ml) containing 50 ml of S-100, 2,421 mg (4.0 mmoles) of disodium ATP, 25 mg (30 μmoles) of CoA, 20 ml of 100 mM magnesium acetate, 20 ml of 600 mM potassium chloride, 20 ml of 100 mM 2-mercaptoethanol and 20 ml of 1M potassium phosphate buffer (pH 7.2). After 3 hour incubation at 37°C, the antibiotic activity assayed by disc-plate method with *Bacillus subtilis* PCI 219 was completely lost.

The reaction mixture was diluted with 200 ml of water and kept in a boiling water bath for 10 minutes. It was filtered and the filtrate was passed through a column of Amberlite CG-50 (NH_4^+ form, 50 ml). After washing with 2,000 ml of water, the inactivated gentamicin C_1 was eluted with 0.1N ammonia. The eluate which was positive in ninhydrin and RYDON-SMITH reactions was concentrated to dryness, yielding 101 mg of a yellowish powder. The powder was subjected to chromatography on Amberlite CG-50 (NH_4^+ form, 20 ml). The purified inactivated gentamicin C_1 was eluted with 0.05N ammonia and obtained as a white powder (86 mg), mp 96~104°C.

On high-voltage paper electrophoresis at 3,000 volts for 20 minutes using formic acid-acetic acid-water (25:75:900 in volume), the inactivated gentamicin C_1 showed a single spot which moved 20.0 cm toward the cathode. Gentamicin C_1 moved 23.0 cm. The IR spectrum of the inactivated gentamicin C_1 showed amide bands I and II (1,650 and 1,570 cm^{-1}). It showed only end absorption in UV spectrum.

In the pmr spectrum of the inactivated gentamicin C_1 in D_2O using tetramethylsilane as the external reference, one N-acetyl signal was seen at δ 2.45 ppm. The signals of 2′-H and 3″-H of both gentamicin C_1 and the inactivated one was shown by INDOR method at the same region, δ 3.3~3.5 ppm, indicating no acetylation on 2′-NH_2 and 3″-NH_2. The high-resolution mass spectrum gave the following signals: *m/e* 520.3331 (6%, $C_{23}H_{46}N_5O_8$, calcd. 520.3343, $[M+1]^+$), 233.1172 (21%, $C_9H_{17}N_2O_5$, calcd. 233.1136, the first fragment from N-acetyldeoxystreptamine moiety), 215.1032 (15%, $C_9H_{15}N_2O_4$, calcd. 215.1031, the second fragment from N-acetyldeoxystreptamine), 205.1196 (23%, $C_8H_{17}N_2O_4$, calcd. 205.1188, the third fragment from N-acetyldeoxystreptamine), 187.1075 (21%, $C_8H_{15}N_2O_3$, calcd. 187.1081, the fourth fragment from N-acetyldeoxystreptamine), 160.0993 (66%, $C_7H_{14}NO_3$, calcd. 160.0973, from garosamine) and 157.1355 (100%, $C_8H_{17}N_2O$, calcd. 157.1340, from purpurosamine). This fragmentation was in good agreement with the fragmentation patterns of aminocyclitol antibiotics reported by DANIELS *et al.*[2], and indicated that one of amino groups of the 2-deoxystreptamine moiety is acetylated.

From the hydrolyzate (6N hydrochloric acid, 100°C, 30 minutes) of the tetra-N-ethoxycarbonyl-derivative of the inactivated gentamicin C_1, levorotatory 1-N-ethoxycarbonyl-2-deoxy-

streptamine (mp 197~201°C, $[\alpha]_D^{28}$ −14° (*C* 0.7, water), *m/e* 235 $[M+1]^+$, the antipode of 3-N-ethoxycarbonyl-2-deoxystreptamine[3]), was isolated by resin chromatography. Consequently, the structure of the inactivated gentamicin C_1 was confirmed to be gentamicin C_1 3-acetate (Fig. 1).

Fig. 1. Gentamicin C_1 3-acetate

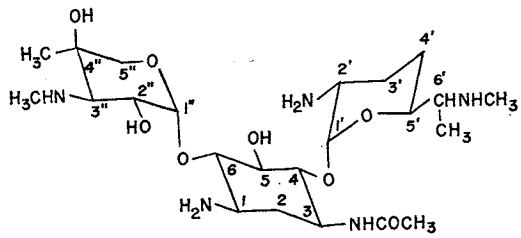

Table 1. Incorporation of ^{14}C-2-acetate into antibiotics by gentamicin acetyltransferase from *E. coli* K12 C600 R135

Antibiotics	Incorporation of ^{14}C-2-acetate (dpm)
Gentamicin C_1	5,523
Gentamicin C_{1a}	7,932
Gentamicin C	6,110
Kanamycin	155
Kanamycin B	290
Kanamycin C	115
3′,4′-Dideoxykanamycin B	207
Ribostamycin	239
Butirosin A	143
Neomycin	176
Paromomycin	123
Lividomycin A	165

Each antibiotic (0.2 μmole) was incubated with the diluted S-100 (190 μg protein from *E. coli* K12 C600 R135) at 37°C for 60 minutes in the reaction mixture (1 ml) containing 16 mM ATP, 0.12 mM CoA, 10 mM magnesium chloride, 60 mM potassium chloride, 10 mM 2-mercaptoethanol, 10 mM sodium acetate, 0.5 μCi ^{14}C-2-sodium acetate (48 μCi/μmole) and 100 mM potassium phosphate buffer (pH 7.2). The mixture was passed through a column of Amberlite CG-50 (NH_4^+ form, 0.5 ml). After washing with 10 ml of water, the radioactivity in the eluate (3 ml) with 0.5 N ammonia was counted in a Beckmann LS-250 scintillation system using BRAY's scintillator.

In another experiment, we studied acetylation of antibiotics in the reaction mixture containing ^{14}C-2-acetate. The reaction product was adsorbed on Amberlite CG-50 resin, and after washing, it was eluted and the radioactivity was counted. The value obtained in the reaction system without the enzyme or without the substrate was taken as the blank, and the mean of these two blanks which were not significantly different was subtracted from the value obtained from the complete system and the counts are recorded in Table 1. As shown by these results, gentamicin C_{1a} and C_1 as well as gentamicin C mixture are the best substrates. The counts observed in reaction mixtures containing 3′, 4′-dideoxykanamycin B, kanamycins, ribostamycin, butirosin A, neomycin, paromomycin and lividomycin A were less than 5% of those observed in the reaction mixture containing gentamicins. Thus, it can be concluded that this enzyme is specific to the 3-amino group of gentamicins, and the result suggests the involvement of the garosamine moiety of gentamicins in binding with the enzyme. A similar enzyme which acetylated the 3-amino group of the deoxystreptamine moiety of gentamicin C components but not of kanamycin, neomycin, paromomycin was found by BRZEZINSKA *et al.*[4] in *Pseudomonas aeruginosa* isolated from patients. MITSUHASHI *et al.*[5] also described a similar enzyme in *P. aeruginosa* 99 which seemed to acetylate the same amino group.[6] However, it is the first time that such an enzyme has been isolated from *E. coli* carrying an R factor and is the confirmation that 3′, 4′-dideoxykanamycin B does not undergo this enzyme reaction. Specific resistance of *E. coli* K12 C600 R135 reported by WITCHITZ[1] can be explained by the formation of gentamicin acetyltransferase described in this paper.

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