ADENYLYLSTREPTOMYCIN, A PRODUCT OF STREPTOMYCIN INACTIVATED BY *E. COLI* CARRYING R FACTOR

Sir:

In previous papers[1,2] reporting phosphorylative inactivation of aminoglycosidic antibiotics by *E. coli* carrying the R factor, phosphorylation of streptomycin was suggested for the mode of inactivation of this antibiotic because of reactivation by alkaline phosphatase of calf mucosa. However, further studies have indicated that the inactivated streptomycin is a compound consisting of adenylic acid and streptomycin. The studies on inactivation of streptomycin by an enzyme preparation from *E. coli* ML 1629 carrying R factor are reported in this paper.

As described in a paper by OKANISHI *et al.*[2], *E. coli* ML1629 was obtained by transmission of R factor from a naturally isolated drug resistant *E. coli* to *E. coli* K 12 ML 1410 which was made resistant to nalidixic acid and shows resistance to kanamycin, kanamycin B, kanamycin C, paromamine, neamine, paromomycin, neomycin, streptomycin, chloramphenicol and tetracycline. The enzyme solution was prepared from cells of this organism in the logarithmic growth phase by the method described by OKANISHI *et al.*[2] The supernatant of the disrupted cells centrifuged at $100,000 \times g$ was treated with 2 mcg/ml of deoxyribonuclease (purchased from Worthington Biochemical Corporation, New Jersey) and 2 mcg/ml of crystalline ribonuclease (purchased from Worthington Biochemical Corporation, New Jersey) at 37°C for 15 minutes. Afterwards it was dialyzed against modified TMK solution (0.06 M KCl, 0.01 M $MgCl_2$ and 0.006 M 2-mercaptoethanol in 0.1 M tris buffer of pH 7.9). The enzyme solution thus prepared was diluted with modified TMK solution to contain 10 mg of protein per ml as determined by the FOLIN method.

The enzyme solution inactivates streptomycin in the presence of ATP and the optimum pH of the inactivation reaction is 8.3~8.5. Marked inactivation, however, also occurs at pH 7.9. The inactivation reaction occurs not only in the presence of ATP but also ADP. However, no reaction occurs with AMP, GTP, UTP or CTP.

In one experiment, 560 mg of streptomycin sulfate was inactivated in 100 ml of the reaction mixture containing 3,600 mg of disodium ATP, 980 mg of $NaHCO_3$, 40 ml of 10 times concentrated modified TMK solution and 30 ml of the enzyme solution. After 20 hours at 37°C inactivation of 80 % of streptomycin was shown by determination of residual streptomycin using a cylinder plate method. *B. subtilis* was used as the test organism. After the reaction, the reaction mixture was passed through a column of 200 ml of Amberlite IRC-50 (Na^+), and the inactivated streptomycin on the column was eluted with 0.5 N HCl. The fraction which gave a positive SAKAGUCHI reaction and which showed no antibacterial activity was collected and passed through a column of active carbon (20 g). The inactivated streptomycin was eluted with 0.2 N HCl-methanol (1 : 1). The fraction giving a positive SAKAGUCHI reaction and negative biological activity was cut and neutralized with Dowex 44 (OH^-). After concentration under vacuum, the inactivated streptomycin was precipitated by addition of 14 volumes of acetone and dried *in vacuo*. A part of the inactivated streptomycin in the reaction mixture passed the column of IRC-50 resin, but it was adsorbed by another column of the same resin and purified by the same procedure as described above. Totally, 250 mg of the inactivated streptomycin was obtained.

The inactivated streptomycin thus obtained showed one spot with the following paper chromatographic systems: Rf 0.18 with water adjusted to pH 10.0 with aqueous ammonia, Rf 0.08 with methanol - ethanol - conc. HCl - water (50 : 25 : 6 : 19), Rf 0 with *n*-butanol saturated with water-28 % NH_4OH (100 : 1), Rf 0 with butanol - acetic acid - water (4 : 1 : 1). Rf values of streptomycin with these solvents were 0.12, 0.20, 0 and 0.02 respectively.

In high voltage paper electrophoresis using acetic acid - formic acid - water (75 : 25 : 900) under 3,500 volts for 15 minutes, the inactivated streptomycin moved to the cathode

by 10.5 cm and streptomycin by 13.0 cm. As shown by the paper electrophoresis study, the inactivated streptomycin is less basic than streptomycin. The presence of phosphorus in the inactivated streptomycin was shown by HANES reagent. The inactivated streptomycin showed a maximum at 260 mμ in the ultraviolet spectrum when it was dissolved in distilled water, 0.1 N HCl or 0.01 N NaOH and the absorption spectrum was similar to that of adenylic acid except for the optical density. Determination of streptomycin by maltol reaction and the optical density at 260 mμ indicated the presence of streptomycin and adenylic acid in the molar ratio of 1 : 1 in the inactivated streptomycin. Anal. calcd. for $C_{21}H_{38}O_{12}N_7 \cdot C_{10}H_{12}O_6N_5PNa \cdot 2HCl \cdot 4H_2O$: C 34.55, H 5.61, O 32.66, N 15.60, P 2.87, Cl 6.58, Na 2.13. Found: C 34.65, H 6.02, O 29.97, N 13.39, P 2.50, Cl 6.04, Na 1.36.

As described in previous papers[1,2], the inactivated streptomycin was reactivated by alkaline phosphatase of calf mucosa (Type 1, purchased from Sigma Chemical Co.). Paper chromatography of the enzymatic hydrolysate using water adjusted to pH 10.0 with aqueous ammonia indicated that the inactivated streptomycin was hydrolyzed to streptomycin (Rf 0.12) and adenosine (Rf 0.55). The identity of the hydrolyzed product with adenosine was proved by paper chromatography with an authentic sample and by the ultraviolet absorption spectrum. The inactivated streptomycin was hydrolyzed to streptomycin and adenylic acid by treatment with phosphodiesterase (venom phosphodiesterase purchased from Worthington Biochemical Corporation). The identity with adenylic acid was proved by paper chromatography using water adjusted to pH 10.0 with aqueous ammonia (Rf 0.95) and isobutyric acid – acetic acid – 1 N NH_4OH (10 : 1 : 5) (Rf 0.69). Rf values of ADP and ATP with the latter solvent were 0.55 and 0.43 respectively. The hydrolysis of the inactivated streptomycin to streptomycin and adenosine by the alkaline phosphatase is considered to be due to contamination with phosphodiesterase in the enzyme employed.

The studies on the inactivated streptomycin as described above indicate that an enzyme in cells of *E. coli* ML1629 carrying R factor catalyzes the reaction of streptomycin and ATP or ADP to produce adenylylstreptomycin. The supernatant of disrupted cells centrifuged at 100,000 × *g* of *E. coli* K12 which is sensitive to streptomycin has no ability to inactivate streptomycin. It suggests that the enzymatic inactivation is the reason for the resistance of *E. coli* carrying R factor. Adenylylation of streptomycin is a new type of inactivation by resistant organisms.

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