PHOSPHORYLATION AND INACTIVATION OF AMINOGLYCOSIDIC ANTIBIOTICS BY *E. COLI* CARRYING R FACTOR

MASANORI OKANISHI, SHINICHI KONDŌ, RYŌZŌ UTAHARA and HAMAO UMEZAWA

Institute of Microbial Chemistry, Tokyo and National Institute of Health, Tokyo

(Received for publication June 23, 1967)

An enzyme solution was prepared by centrifugation at 100,000×g of disrupt cells of *E. coli* K 12 ML 1629 carrying R factor. It inactivated kanamycin, streptomycin and paromamine. This enzyme solution required ATP and Mg^{++} for inactivation of these antibiotics, but not coenzyme A and acetate. One of the inactivation products of kanamycin recovered activity by treatment with alkaline phosphatase. The activity of inactivated paromamine was restored by the alkaline phosphatase. Thus, the inactivation was suggested to be due to phosphorylation. The enzyme solution prepared from the sensitive strain, *E. coli* K 12 (CS-2) and its subcultures which had been made resistant by transfers in kanamycin medium showed no inactivation of kanamycin, and thus the formation of enzymes for inactivation was characteristic of the strain carrying R factor. Alkaline phosphatase formation in *E. coli* K 12 ML 1629 is repressed by phosphate at a lower concentration than that in *E. coli* K 12 (CS-2). The enzyme solution prepared from *E. coli* K 12 (CS-2) which grew under conditions repressing formation of alkaline phosphatase showed no inactivation of kanamycin. The sensitivity of *E. coli* K 12 (CS-2) and *E. coli* K 12 ML 1629 to kanamycin was not influenced by the presence or absence of alkaline phosphatase repression. It was shown that phosphorylative inactivation is one of the mechanisms of resistance of *E. coli* carrying R factor.

OKAMOTO and SUZUKI[1] found that a multiply drug resistant *E. coli* K 12 (R-5) produced an enzyme inactivating kanamycin. The inactivated kanamycin was isolated by UMEZAWA *et al.*[2] and found to be N-acetylkanamycin in which amino group of 6-amino-6-deoxy-D-glucose moiety was acetylated. As reported in another paper[3], this resistant strain of *E. coli* is sensitive to kanamycin C and kanamycin C is not inactivated by the enzyme solution. Therefore, the inactivation of the antibiotic must be directly related with the resistance.

E. coli K 12 ML 1629 carrying another R factor is highly resistant to kanamycin, kanamycin C, paromomycin and neomycin. The cross resistance to these antibiotics indicates that the mechanism of the resistance of this strain is different from that of *E. coli* K 12 (R-5). We found that this highly resistant *E. coli* carrying R factor produced an enzyme with weak kanamycin inactivating activity when cultured at 37°C but produced a strongly active enzyme solution when cultured at 27°C. We studied the conditions necessary for kanamycin inactivation, and proved that the inactivation was due to phosphorylation of kanamycin. The structure of the inactivated product is reported in another paper[4]. This strain of *E. coli* is also

resistant to dihydrostreptomycin and produces an enzyme solution inactivating this antibiotic. On the other hand, a subculture of *E. coli* K 12 (CS-2) which was made resistant to kanamycin by the successive transfers in a medium containing kanamycin did not produce an enzyme solution inactivating kanamycin. Thus, this phosphorylative inactivation is related to the resistance exhibited by this R factor. We now add a new mechanism of inactivation by enzymes of resistant strains, that is phosphorylation, to the known mechanisms, acetylation observed with chloramphenicol and kanamycin and hydrolysis of β-lactam linkage observed in the case of penicillin.

In this paper, we report our studies on conditions necessary for inactivation of kanamycin, paromamine, and dihydrostreptomycin by an enzyme solution obtained from *E. coli* K 12 ML 1629, on reactivation of inactivated antibiotics by alkaline phosphatase, on the negative effect of an enzyme solution obtained from *E. coli* K 12 (CS-2) made resistant to kanamycin by the successive transfers, and on the formation of alkaline phosphatase in the resistant or sensitive cells of *E. coli*.

Materials and Methods

Strains employed: *E. coli* K 12 ML 1629 and *E. coli* K 12 ML 1630 were supplied by Prof. S. MITSUHASHI, Department of Bacteriology, Medical School, Gumma Universiy. According to him, these strains were obtained by transmission of R factor from a naturally isolated drug resistant *E. coli* to *E. coli* K 12 ML 1410 which was resistant to nalidixic acid. *E. coli* K 12 (R-5) was supplied by Dr. OKAMOTO, Department of Chemistry, National Institute of Health, Tokyo. It is a five-drug resistant strain of *E. coli* K 12 (CS-2) obtained by transmission of R factor (R-5) from a naturally isolated drug resistant *Shigella sonnei*. *E. coli* K 12 (CS-2) is the sensitive strain. *Shigella sonnei* (191-66) was obtained from Dr. NAKAYA, Department of Bacteriology, National Institute of Health, Tokyo. It was isolated from a patient in Niigata Prefectural Institute of Health.

Preparation of the enzyme solution: *E. coli* K 12 ML 1629 was cultured in 100 ml of peptone broth (glucose 1 g, peptone 10 g, 0.01 M $CaCl_2$ 10 ml, 0.1 M $MgSO_4$ 10 ml, 0.1 M KH_2PO_4 3.2 ml, NaCl 3 g, 1 N NaOH 2.5 ml and the total volume made to 1,000 ml with distilled water) under agitation by a magnetic stirrer at 27°C for 20 hours. The cultured broth was transferred to 900 ml of peptone broth containing kanamycin at 5 mcg/ml and incubated under aeration at 27°C for 7 hours. It was then chilled rapidly by pouring on crushed ice and the cells were collected by centrifugation (6,000×g, 20 minutes). The sedimented cells were washed twice with 1 L of modified TMK solution (0.06 M KCl, 0.01 M $MgCl_2$ and 0.006 M 2-mercaptoethanol in 0.1 M *tris* buffer).

The cells were suspended in modified TMK solution in a volume equal to the cell pellet and disrupted by passage through a French pressure cell (1,500 kg/cm^2). The suspension of disrupted cells was centrifuged at 30,000×g for 20 minutes and the supernatant was centrifuged at 100,000×g for 90 minutes. The supernatant thus obtained was dialyzed overnight against modified TMK solution. The retentate obtained after the dialysis was designated the enzyme solution. The enzyme solution was diluted to 10 mg/ml of protein determined by FOLIN method. The enzyme solutions of *E. coli* K 12 (R-5) and the subculture of *E. coli* K 12 (CS-2) made resistant in the laboratory were prepared by the same procedure as described above.

Antibiotic inactivation reaction: The reaction mixture contained the following materials: 0.6 ml of 10 times concentrated modified TMK solution, 48 mg (80 μ moles) of disodium ATP, 13 mg (155 μ moles) of $NaHCO_3$, 10 μ moles of the antibiotic (that is, 7 mg of kanamycin sulfate), 0.3 ml of the diluted enzyme solution. The total volume was made

to 1.0 ml with distilled water. The reaction was run at 37°C for 3~20 hours. In the case of dihydrostreptomycin, 100 mcg of this antibiotic (0.14 μ mole) and 3 mg of ATP (4 μ moles) were added to reaction mixture.

Determination of alkaline phosphatase in cells: The enzyme activity wes determined by the method described by ECHOLS *et al.*[5] using *p*-nitrophenyl phosphate as the substrate and the unit was expressed as the change in the optical density at 410 mμ/min./cell density at 540 mμ. The cells on which the activity was determined were grown in LEVINTHAL'S peptone broth[6] in which the concentration of phosphate was varied.

Growth inhibitory activity: The minimum inhibitory concentrations of antibiotics were determined by an agar dilution method using heart infusion medium. A test organism was cultured in heart infusion broth at 27°C overnight. The cultured broth was diluted 500 times with the same medium and one loopful was streaked on the agar medium containing the antibiotic and incubated for 2 days.

Materials: Kanamycin sulfate, kanamycin C and dihydrostreptomycin sulfate were obtained from Meiji Seika Co. and paromamine was prepared in the authors' laboratory from paromomycin. Paromomycin and neomycin were obtained in the market. Alkaline phosphatase of calf mucosa Type I and phosphodiesterase of *Crotalus admanteus* venom Type II were purchased from Sigma Chemical Company.

Results

1. The inhibitory concentrations of kanamycin, tritiated kanamycin, kanamycin C, 6-amino-6-deoxy-D-glucosyldeoxystreptamine obtained from kanamycin by a controled hydrolysis, paromamine, neamine, paromomycin, neomycin, dihydrostreptomycin, destomycin, chloramphenicol and tetracycline to *E. coli* K 12 (CS-2), *E. coli* K 12 (R-5), *E. coli* K 12 ML 1629, *E. coli* K 12 ML 1630 and *Shigella sonnei* (191-66) are indicated in Table 1. *E. coli* K 12 (CS-2), the sensitive strain, is inhibited by all of these antibiotics or hydrolysis products of kanamycin, paromomycin and neomycin. K 12 (R-5) is resistant to kanamycin and 6-amino-6-deoxy-D-glucosyldeoxystreptamine, but sensitive to kanamycin C, paromamine and paromomycin which do not contain 6-amino-6-deoxy-D-glucose. As reported in another paper[2], this strain K 12 (R-5) produces an enzyme acetylating the amino group of 6-amino-6-deoxy-D-glucose moiety of kanamycin. Strain (R-5) is resistant to neamine but sensitive to kanamycin B and neomycin. These compounds do not contain 6-amino-6-deoxy-D-glucose but contain 2,6-diamino-2,6-dideoxy-D-glucose. The resistance to neamine and the

Table 1. Minimum inhibitory concentrations of various aminoglycosidic antibiotics against *E. coli* and *S. sonnei*

Antibiotic	MIC (mcg/ml)				
	CS-2*	R-5 *	ML1629*	ML1630*	191-66 *
Kanamycin	1.25	10~20	>320	>320	>320
Kanamycin C	5	5	>320	>320	>320
Kanamycin B	2.5	2.5	>320	>320	>320
^{3}H-Kanamycin	1.25	10	>320	>320	>320
6AG-DSM*	40~80	>320	>320	>320	>320
Paromamine	40	10	320	>320	>320
Neamine	10	160	>320	>320	>320
Paromomycin	1.25	1.25	>320	>320	>320
Neomycin	1.25	1.25	>320	>320	>320
Dihydrostreptomycin	2.5	20	20	20	20
Destomycin	40	5	20~40	20~40	20
Chloramphenicol	2.5	>320	>320	>320	>320
Tetracycline	2.5	80	80	2.5	160

* Abbreviations : CS-2 : *E. coli* K12 (CS-2). R-5 : *E. coli* K12 (R-5). ML1629 : *E. coli* K12 ML1629. ML1630 : *E. coli* K12 ML1630. 191-66 : *Shigella sonnei* (191-66). 6AG-DSM : 6-amino-6-deoxy-D-glucosyldeoxystreptamine obtained by hydrolysis of kanamycin.

sensitivity to kanamycin B and neomycin suggest that the amino group on C-6 of the 2, 6-diamino-2, 6-dideoxy-D-glucose moiety in kanamycin B and neomycin would not be acetylated or the acetylated kanamycin B and neomycin are active against K12 (R-5). The strain (R-5) carries resistance to chloramphenicol, tetracycline and dihydrostreptomycin. Though the reason is not clear, the strain K12 (R-5) is more sensitive to paromamine and destomycin than K12 (CS-2). Destomycin[7] is an antibiotic closely related to hygromycin B and does not contain 6-amino-6-deoxy-D-glucose. It was recently reported to inhibit protein synthesis in a cell free system and to cause misreading of codes.

K12 ML1629, K12 ML1630 and *S. sonnei* (191-66) are highly resistant to deoxystreptamine-containing antibiotics and sensitive to destomycin which contains N-methyldeoxystreptamine. K12 ML1629 and *S. sonnei* (191-66) are resistant to tetracycline, but K12 ML1630 is sensitive. All three strains show moderate resistance to dihydrostreptomycin.

2. Relation of resistance to incubation temperatures: K12 (CS-2), K12 (R-5), K12 ML1629 and *S. sonnei* (191-66) were cultured at varied temperatures in heart infusion broth for 20 hours and the cultured broths were diluted and spread on heart infusion agar plates containing kanamycin at 0, 20, 40, 80 and 160 mcg/ml. After incubation at 27°C for two days, the number of colonies were counted. The result is shown in Table 2. K12 (CS-2) gave no colonies on plates containing not less than 20 mcg/ml of kanamycin. In all cases, the ratio of numbers of resistant cells to whole cells was larger when cultured at the lower temperature. Especially, when K12 ML1629 was cultured at 45°C, the ratio of the resistant cells to whole cells was less than half of that observed when incubated at 27°C or 37°C. The number of resistant cells was smaller also in the case of *S. sonnei* when cultured at 37°C than when cultured at 27°C. This difference was more marked at 80 or 160 mcg/ml of kanamycin.

The ratio of resistant cells to whole cells of K12 ML1629 was not markedly different when the strain was cultured at 27°C or at 37°C, but the enzyme solution prepared from cells cultured at 27°C was much more active in inactivating kanamycin than that prepared from cells cultured at 37°C.

Table 2. Ratio of resistant cells to whole cells obtained by preincubation at varied temperature

	Temp. of paeincubation (°C)	Percent *				
		0 mcg/ml	20 mcg/ml	40 mcg/ml	80 mcg/ml	160 mcg/ml
S. sonnei (191-66)	27	100	102	97	110	98
〃	37	100	84	44	30	23
E. coli ML1629	27	100	104	100	96	94
〃	37	100	110	92	94	90
〃	45	100	45	40	25	18
E. coli K12 (R-5)	27	100	5	5	0	0
〃	37	100	3	2	0	0
E. coli K12 (CS-2)	27	100	0	0	—	—
〃	37	100	0	0	—	—

* Percent of number of colonies on a plate to that of a plate without the antibiotic.

3. Conditions for kanamycin inactivation: The culture filtrate of K12 ML1629 and K12 ML1630 showed no kanamycin inactivating activity, even though ATP, CoA and acetate were added. An enzyme solution inactivating kanamycin was obtained only from disrupted cells.

As reported in a previous paper[3], the enzyme solution obtained from K12 (R-5) acetylates and inactivates kanamycin in the presence of ATP, CoA and acetate. The enzyme solution obtained from K12 ML1629 inactivated kanamycin in the same reaction mixture. However, the results of experiments removing each component indicated that coenzyme A, acetate and ATP generators were not necessary for inactivation of kanamycin by the enzyme solution of K12 ML1629, but ATP and Mg^{++} were necessary. As shown in Table 3, the concentrations of ATP necessary for inactivation of 5,000 mcg/ml (0.01 M) of kanamycin or 500 mcg/ml (0.001 M) of kanamycin were as follows: when 0.04 M ATP was added to the reaction mixture containing 0.01 M kanamycin, 64 % of the kanamycin was inactivated after 20 hours of the reaction; when 0.08 M ATP was added, 97 % of the kanamycin was inactivated; when kanamycin was added at 0.001 M, the addition of ATP at 0.008 M caused more than 90 % inactivation. Thus, about 8 moles of ATP were necessary for inactivation of 1 mole of kanamycin. However the enzyme solution was found to contain enzymes converting ATP to adenosine. When ATP was added at 0.08 M to the reaction mixture without the antibiotic, 90 % of ATP was shown by a thin-layer chromatography to be converted to adenosine after 6 hours reaction. After 24-hour reaction, ATP was further degraded to adenine. Magnesium ion was found to be necessary for inactivation of kanamycin. As shown in Table 4, when kanamycin at 0.001 M and ATP at 0.008 M were added, addition of 0.003~0.033 M $MgCl_2$ caused inactivation of more than 80 % of kanamycin. When kanamycin at 0.01 M and ATP at 0.08 M were added, addition of 0.003 M magnesium chloride caused inactivation of

Table 3. ATP for inactivation of kanamycin

ATP (M)	Residual kanamycin (mcg/ml)		Inactivated kanamycin (%)
	0 hr.	24 hrs.	
0	5,000*	4,800	4
0.01	5,000	4,400	12
0.02	5,000	2,400	52
0.04	5,000	1,800	64
0.08	5,000	120	97
0	480**	400	17
0.001	480	320	33
0.002	480	280	42
0.004	480	170	65
0.008	480	40	90

* 5,000 mcg/ml=0.01 M
** 480 mcg/ml=0.001 M

In addition to ATP, $NaHCO_3$ and KM, the reaction mixture contained ten time-concentrated modified TMK solution 0.6 ml and 100 S fraction 0.3 ml in a total volume of 1.0 ml with distilled water.

Table 4. Concentration of $MgCl_2$ in relation to concentrations of kanamycin

$MgCl_2$ (M)	Residual kanamycin (mcg/ml)		Inactivated kanamycin (%)
	0 hr.	20 hrs.	
0.003	480	96	80
0.009	480	24	95
0.033	480	0	100
0.063	480	15	97
0.003	5,000	4,000	20
0.009	5,000	1,750	65
0.033	5,000	0	100
0.063	5,000	0	100

* 8 mM of ATP in the case of 480 mcg/ml of kanamycin and 80 mM of ATP in the case of 5,000 mcg/ml of kanamycin.

20 % of kanamycin added. When $MgCl_2$ was added at 0.033 M or 0.063 M, 100 % of the kanamycin was inactivated. In another experiment, in the reaction mixture containing kanamycin at 0.001 M and Mg^{++} at 0.003 M, addition of calcium chloride, cobalt chloride or barium chloride was inhibitory for the inactivation reaction. Addition of manganese chloride or zinc chloride showed no promotion of inactivation of kanamycin. Thus, magnesium ion can not be replaced by other divalent ions.

Twenty L-amino acids except serine and combination of cofactors such as NAD, NADP, FAD, FMN, pyridoxal phosphate, thiamine pyrophosphate, folic acid, biotin, *p*-aminobenzoic acid, lipoic acid and α-ketoglutarate were studied for their influence on the inactivation reaction, but none of them gave any influence on the inactivation reaction. Addition of DNAase and RNAase to the enzyme solution showed no inactivation of the enzyme activity.

ATP was not replaced by ADP, AMP, CTP, GTP or UTP. Thus, it is concluded that the enzyme solution obtained from K12 ML1629 requires ATP and Mg^{++} for inactivation of kanamycin.

4. Inactivation of paromamine: When 0.01 M of paromamine was used in place of kanamycin, more than 90 % of the paromamine was inactivated after 20-hour reaction at 37°C.

5. Inactivation of dihydrostreptomycin: Enzyme solutions were prepared from K12 (R-5) and K12 ML1629 and the inactivation reaction was carried under the conditions described in the section on Materials and Methods. One hundred % of dihydrostreptomycin was 100 % inactivated by these enzyme solutions. The addition of coenzyme A and acetate was not necessary for inactivation of dihydrostreptomycin. ATP and probably Mg^{++} were found to be necessary for inactivation of dihydrostreptomycin.

6. Reactivation of inactivated products of kanamycin, dihydrostreptomycin and paromamine by alkaline phosphatase: As reported in another paper[4], an inactivated kanamycin and an inactivated paromamine were isolated and were proved to be the phosphorylated derivatives, in which the hydroxyl group on C-3 of 6-amino-6-deoxy-D-glucose or D-glucosamine (2-amino-2-deoxy-D-glucose) was phosphorylated. The effect of alkaline phosphatase or of phosphodiesterase on these inactivated derivatives was studied. The reaction mixture contained 0.1 ml of the solution of an inactivated antibiotic (1 mg/ml), 0.8 ml of BMK solution (0.05 M KCl, 0.01 M $MgCl_2$ in 0.05 M borate buffer of pH 10.0) and 0.1 ml of an enzyme solution (5 mcg/ml of alkaline phosphatase and 2 mcg/ml of phosphodiesterase). After reaction at 37°C for 1~6 hours, the reaction was stopped by the ten-fold dilution with 0.1 M phosphate buffer (pH 7.0) and the activity against *B. subtilis* was tested by a cylinder plate method. The inactivated kanamycin corresponding to 80 mcg of kanamycin base recovered 64 mcg of kanamycin activity after 1 hour and 70 mcg after 3 or 6 hours. Eighty mcg of inactivated paromamine gave 75 mcg of paromamine activity after 3 hours of reaction. These recovery of activity by the treatment of alkaline phosphatase was completely inhibited by the presence of inorganic phosphate. The inactivated antibiotics did not recover any activity by reaction with phosphodiesterase.

Dihydrostreptomycin (100 mcg/ml) was inactivated by the enzyme solution obtained from K12 (R-5) or K12 ML1629. To 1 ml of the reaction mixture after 20 hours reaction, 0.5 mcg of an alkaline phosphatase was added, and 100 % of the activity was recovered after the incubation at 37°C for 5 hours. Thus, dihydrostreptomycin inactivated by enzyme solution obtained from both strains of *E. coli* was suggested to be a phosphorylated derivative.

7. Inactivity of the enzyme solution prepared from K12 (CS-2) made resistant in the laboratory. K12 (CS-2) was spread on plates containing kanamycin at 5, 10, 20, 40, 80, 160 mcg/ml. No colonies were observed on plates containing kanamycin at 10 mcg/ml or more, but a few colonies were observed on the plate with 5 mcg/ml. From one of these colonies a culture resistant to 5 mcg/ml was obtained. In another experiment, K12 (CS-2) was transferred successively through heart infusion broth containing kanamycin at increasing concentrations. During the successive transfers, the concentration of kanamycin was raised and a subculture which grew in the medium containing 80 mcg/ml of kanamycin was obtained. The cells of these two resistant strains were disrupted and the two enzyme solutions were prepared by the method described. These enzyme solutions were found to produce no inactivation of kanamycin.

8. Production of alkaline phosphatase by K12 (CS-2) and K12 ML1629 in relation to concentration of phosphate: These two strains were shaken in LEVINTHAL's peptone broth[6] containing potassium phosphate at 5×10^{-4} M at 37°C overnight and 0.1 ml of the cultured broth was inoculated into 10.0 ml of LEVINTHAL's peptone medium containing varied concentrations of potassium phosphate and cultured again overnight at 37°C. The cells were collected by centrifugation and suspended in 0.1 M *tris* buffer, the optical density of the suspension was adjusted to give 1.0 at 540 mμ. After the addition of toluene, it was shaken for 1 hour at 37°C. After centrifugation, the alkaline phosphatase activity of the supernatant was determined by a method described by ECHOLS *et al.*[5] *p*-Nitrophenylphosphate was used as the substrate; the unit was defined as follows: change in optical density (10 mm cuvette) at 410 mμ/minute/optical density of cell at 540 mμ. The result is shown in Table 5. Both strains were found to produce 100 units of alkaline phosphatase in a medium containing inorganic phosphate at 5×10^{-5} M. K12 (CS-2) also produced 100 units in a medium with 2.5×10^{-4} M phosphate but K12 ML1629 produced only 1.6 units in the medium of the same phosphate concentration. Thus, production of alkaline phosphatase of K12 ML1629 was repressed by a lower concentration of phosphate than K12 (CS-2).

Table 5. Alkaline phosphatase activity in cells of *E. coli* K12 (CS-2) and *E. coli* K12 ML1629 grown in LEVINTHAL's medium with varied concentration of phosphate

Inorganic phosphate (M)	Activity of alkaline phosphatase (unit)	
	CS-2 *	ML1629 *
10^{-3}	0	0
5×10^{-4}	8.7	1.1
2.5×10^{-4}	100	1.6
5×10^{-5}	100	100

* shown in Table 1.

9. The inhibitory concentration of kanamycin against K12 (CS-2) and K12 ML1629 with or without repression of alkaline phosphatase formation: The inhibitory concentrations of kanamycin on K12 ML1629 and K12 (CS-2) were tested in

LEVINTHAL's medium containing phosphate at varied concentration. The inoculum was prepared in LEVINTHAL's medium containing phosphate at the same concentration as the test medium. After 20 hours incubation, the inhibitory concentrations of kanamycin against both strains of *E. coli* was examined. The results indicated no difference related to the concentrations of phosphate. K12 ML1629 was inhibited at 2,560 mcg/ml of kanamycin in LEVINTHAL's medium in all concentrations of inorganic phosphate. K12 (CS-2) was inhibited at 1.25 mcg/ml of kanamycin in LEVINTHAL's medium and the inhibitory concentration was not influenced by the phosphate concentrations.

Discussion

As reported previously[2,3], *E. coli* K12 (R-5) produces an enzyme acetylating the amino group of 6-amino-6-deoxy-D-glucose moiety of kanamycin and this acetylated product has no antibacterial activity. This organism is sensitive to kanamycin C which has glucosamine instead of 6-amino-6-deoxy-D-glucose and the enzyme obtained from K12 (R-5) does not inactivate kanamycin C. Therefore, the formation of the enzyme acetylating kanamycin is directly related to the resistance to kanamycin. The sensitivity of this strain to paromamine and paromomycin is also explained by the absence of 6-amino-6-deoxy-D-glucose. In these substances, the aminohexose linked to deoxystreptamine is D-glucosamine. K12 (R-5) is resistant to neamine which consists of 2,6-diamino-2,6-dideoxy-D-glucose and deoxystreptamine. This resistance suggests that the amino group on C-6 of 2,6-diamino-2,6-dideoxy-D-glucose in neamine would be acetylated by the enzyme produced by K12 (R-5). Neamine is a constituent of neomycins and kanamycin B, but K12 (R-5) is sensitive to neomycin and kanamycin B. This sensitivity suggests two possibilities: the neamine moiety in neomycin and kanamycin B is not acetylated by the enzyme produced by K12 (R-5) or the acetylated derivatives of neomycin and kanamycin are active against K12 (R-5).

OKAMOTO and SUZUKI[1] reported that K12 (CS-2), the sensitive strain, produced no enzyme inactivating chloramphenicol, but K12 (R-5) obtained by transmission of R factor from a naturally isolated multiple drug resistant *S. sonnei* produced an enzyme acetylating and inactivating chloramphenicol. This observation and our observation on K12 (R-5) indicate that R factor in these strains exhibit the resistance to chloramphenicol and kanamycin by the formation of enzymes acetylating and inactivating these antibiotics.

The acetylating enzyme requires acetate, coenzyme A and ATP. OKAMOTO and SUZUKI[1] reported that the inactivation of dihydrostreptomycin by the enzyme solution obtained from *E. coli* (R-4) did not require coenzyme A and acetate, and the inactivation system for dihydrostreptomycin described by these authors contained ATP. The minimum inhibitory concentration of dihydrostreptomycin against K12 (R-5) is 20 mcg/ml. The enzyme solution obtained from this strain inactivates dihydrostreptomycin in the presence of ATP and the inactivated streptomycin recovers activity by treatment with alkaline phosphatase. The same was observed in the case of K12 ML1629 which requires 20 mcg/ml of dihydrostreptomycin for inhibition. The recovery of activity by the alkaline phosphatase and the requirement of ATP for the inactivation suggests that the inactivation of dihydrostreptomycin by the enzyme obtained from K12 (R-5) and K12 ML1629 is due to the reaction of the antibiotic with ATP.

K12 ML1629 was obtained by transmission of R factor from a naturally resistant *E. coli* to a subculture of *E. coli* K12 made resistant to nalidixic acid. This strain is highly resistant to kanamycin, kanamycin C, paromomycin and neomycin. In the high resistance to these deoxystreptamine-containing antibiotics, K12 ML1629 is different from K12 (R-5). Therefore, it is thought that the mechanism of resistance of these strains is different.

The enzyme obtained from K12 ML1629 requires ATP and Mg^{++} for inactivation of kanamycin and paromamine. The enzyme solution employed was dialyzed and addition of coenzyme A, acetate, amino acids, cofactors such as NAD, FAD and pyridoxalphosphate *etc.* did not show any promotion of the inactivation reaction. The requirement for ATP and Mg^{++} suggests that the inactivation is due to phosphorylation of antibiotics. Most of the activity after the inactivation of kanamycin is recovered by alkaline phosphatase, if the concentration of ATP is low in the reaction mixture. It is not recovered by treatment of the reaction mixture with phosphodiesterase. An inactivated kanamycin isolated, as reported in another paper[4], is phosphorylated on hydroxyl group at C-3 of 6-amino-6-deoxy-D-glucose moiety. The activity of this compound is recovered by treatment with alkaline phosphatase. As also reported in the same paper, in an inactivated product of paromamine, the hydroxyl group on C-3 of glucosamine moiety is phosphorylated. The activity of the inactivated kanamycin and the inactivated paromamine are recovered by treatment with alkaline phosphatase. It is not certain whether the phosphorylation of the hydroxyl group on C-3 of 6-amino-6-deoxy-D-glucose of kanamycin and that on hydroxyl group on C-3 of D-glucosamine of paromamine is catalyzed by the same enzyme or different enzymes. The addition of D-glucosamine or methyl 6-amino-6-deoxy-α-D-glucoside at 0.01 M to the reaction mixture containing kanamycin at 0.001 M did not show inhibition of the inactivation of kanamycin when the reaction was stopped at 3 hours or at 20 hours. Two subcultures of K12 (CS-2), which were made resistant in the laboratory and carry no R factor, give no enzyme solutions inactivating kanamycin. It suggests that the phosphorylation and inactivation of kanamycin is directly related to the resistance. However, it is necessary to know the relation of alkaline phosphatase and the inactivated product. The formation of alkaline phosphatase in cells of both resistant and sensitive strains is repressed by phosphate in the medium. The phosphate concentration repressing the formation of this enzyme is lower in the resistant strain than in the sensitive strain. No difference was found in the inhibitory concentrations of kanamycin for cells with repressed or unrepressed alkaline phosphatase formation. Moreover, an enzyme solution obtained from cells of a sensitive strain, which was incubated with repression of alkaline phosphatase showed no inactivation of kanamycin in the presence of ATP and Mg^{++}. Therefore it is conclusive that the enzyme phosphorylating kanamycin is produced only by *E. coli* carrying R factor.

The fact that enzymes acetylating or phosphorylating kanamycin are obtained from *E. coli* carrying R factor, but not from sensitive or resistant strains without R factor indicates that the inactivation by these enzymes is the mechanism of the resistance.

References

1) Okamoto, S. & Y. Suzuki : Chloramphenicol-, dihydrostreptomycin-, and kanamycin-inactivating enzymes from multiple drug-resistant *Escherichia coli* carrying episome 'R'. Nature 208:1301～1303, 1965

2) Umezawa, H. ; M. Okanishi, R. Utahara, K. Maeda & S. Kondō : 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) Okanishi, M. ; S. Kondō, Y. Suzuki, S. Okamoto & H. Umezawa : Studies on inactivation of kanamycin and resistances of *E. coli*. J. Antibiotics, Ser. A 20 : 132～135, 1967

4) Kondō, S.; M. Okanishi, R. Utahara, K. Maeda & H. Umezawa : Isolation of kanamycin and paromamine inactivated by *E. coli* carrying R factor. J. Antibiotics 21 : 14～22, 1968.

5) Echols, H.; A. Garen, S. Garen & A. Torriani : Genetic control of repression of alkaline phosphatase in *E. coli*. J. Mol. Biol. 3 : 425～438, 1961

6) Levinthal, C.; E. R. Signer & K. Fetherolf : Reactivation and hybridization of reduced alkaline phosphatase. Proc. Natl. Acad. Sci. 48 : 1230～1237, 1962

7) Kondō, S.; E. Akita & M. Koike : The structure of destomycin A. J. Antibiotics, Ser. A 19 : 139～140, 1966