known (*17*). Peroxidase is unique in this respect only in that it seems to possess one function, that of an IAA oxidase, in the nonheme protein portion of the molecule, and a second function, involving guaiacol peroxidation, which depends on a heme-protein attachment. These sites appear to be distinct, since readdition of heme to apoprotein has no effect on the IAA-oxidase activity of the latter.

Peroxidase contains a sizable carbohydrate component (about 15 percent of its dry weight) which remains associated with the protein fraction during these experiments (*18*). Other investigators (*5*) suggested a second active site (the heme-protein complex generally being considered the primary site) of peroxidase which may involve the carbohydrate-protein complex.

The cyanide inhibition of a nonheme-dependent reaction can be explained on the basis that CN^- is an effective free-radical chain terminator (*19*)—some oxidase and peroxidase reactions are believed to function by means of such a mechanism (*20*)—or by the fact that CN forms a complex with Mn^{++} at the active site. The azide effect may be more difficult to understand, although Weinryb (*15*) demonstrated a reaction between azide and amino acid residues which alters the environs of the catalytic center in horseradish peroxidase. Chance (*21*), from his studies on azide inhibition of other hemoprotein systems, concluded that the azide may be capable of attachment only during a transition between two different configurations of the protein, possibly at a histidyl locus. It is generally assumed that azide attaches as a ligand to the heavy metal of the prosthetic group. In our purified apoprotein preparation, there is no detectable iron; nevertheless, Mn^{++} is required for the apoprotein to function as an IAA oxidase, although Mn^{++} cannot replace the heme for peroxidase activity. It is possible that the azide might block the active site formed between the apoprotein and Mn^{++} and hence could act in the classic manner. The *p*H effects may well be due to conformational changes of the protein produced by the charge alteration, as well as reflecting the acid-base properties of the substrates.

The nonperoxidase IAA oxidase reported by Sequeira and Mineo (*9*) may be an artifact resulting from the removal of the heme during the processes involved in isolation. Alternatively, the apoprotein may exist in the free form in vivo. We have attempted to detect free apoperoxidase protein in pea tissue homogenate, but without success.

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Phosphorylative Inactivation of Aminoglycosidic Antibiotics by Escherichia coli Carrying R Factor

Abstract. *Kanamycin and paromamine are inactivated by an enzyme solution prepared from* Escherichia coli *carrying R factor; and the 3-hydroxyl group of 6-amino-6-deoxy-*D*-glucose moiety of kanamycin or the 3-hydroxyl group of glucosamine moiety of paromamine is phosphorylated. These inactivated products and dihydrostreptomycin inactivated by the enzyme solution are converted to the original antibiotics by treatment with alkaline phosphatase.*

Okamoto and Suzuki (*1*) reported that a multiple drug-resistant *Escherichia coli* K12 (R-5) produced an enzyme that inactivates kanamycin. The strain used by these authors was obtained by transmission of R factor (R-5) from a naturally isolated drug-resistant strain of *Shigella sonnei* which was resistant to chloramphenicol, tetracycline, dihydrostreptomycin, sulphanilamides, and kanamycin. These authors suggested that this resistant strain would produce an enzyme that acetylates kanamycin, because of necessity of coenzyme A, acetate, and adenosine triphosphate for the inactivation. As reported earlier, we isolated the inactivated kanamycin and confirmed the fact that amino group of 6-amino-6-deoxy-D-glucose moiety of kanamycin was acetylated. The relation of inactivation to resistance can be shown by the fact that this resistant organism is sensitive to kanamycin C which contains D-glucosamine instead of 6-amino-6-deoxy-D-glucose, and that the enzyme extracted from this organism does not inactivate kanamycin C. We later found that the mode of the inactivation by *E. coli* K12 carrying another R factor was different. We now report on the phosphorylation and inactivation of kanamycin, paromamine, and dihydrostreptomycin by an enzyme solution prepared from a resistant strain of *E. coli* carrying R factor.

Escherichia coli K12 ML 1629 was obtained by transmission of R factor from a naturally isolated drug-resistant strain of *E. coli* to *E. coli* K12 ML 1410 which was resistant to nalidixic acid. The inhibitory concentrations of kanamycin, paromomycin, and neomycin toward *E. coli* ML 1629 were higher than 320 μg/ml, that of paromamine was 320 μg/ml, and that of streptomycin was 20 μg/ml. The inhibitory concentrations for *E. coli* K12 were as follows: kanamycin, 1.25 μg/ml; paromamine, 40 μg/ml; par-

omomycin, 1.25 μg/ml; neomycin, 1.25 μg/ml; and streptomycin, 2.5 μg/ml. When an enzyme solution was prepared from *E. coli* ML 1629 cultured at 37°C, the enzyme solution inactivated kanamycin poorly. We cultured this organism at 27°C and obtained the active enzyme solution. The heating at 80°C for 5 minutes destroyed the enzyme activity to inactivate kanamycin.

The active enzyme solution was prepared as follows. The cells were collected during the last part of the logarithmic phase of growth of *Escherichia coli* ML 1629 grown in shake cultures for 7 hours at 27°C in a peptone medium (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; 1*N* NaOH, 2.5 ml; NaCl, 3 g; and distilled water, 1 liter) and the cells were collected by centrifugation (6000*g*, 20 minutes). The sedimented cells were washed with 1 liter of modified TMK solution (0.06*M* KCl, 0.01*M* $MgCl_2$, and 0.06*M* 2-mercaptoethanol in 0.1*M* tris buffer, *p*H 7.8) by centrifugation. 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 (1500 kg/cm²). The suspension of the 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. This solution was designated the enzyme solution and its protein content, shown by the Folin method, was adjusted to 10 mg/ml.

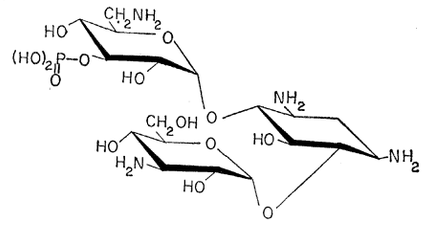
The reaction mixture consisted of the following materials: 0.6 ml of modified TMK solution concentrated tenfold, 48 mg (80 μmole) of disodium adenosine triphosphate with necessary amount of $NaHCO_3$ for the dissolution, 7 mg (10 μmole) of kanamycin sulfate or 4.4 mg (10 μmole) of paromamine and 0.3 ml of the enzyme solution in a total volume adjusted to 1.0 ml with distilled water. In some experiments, instead of tris buffer, 0.025*M* phosphate buffer (*p*H 7.8) was used. The reaction mixture was incubated for 20 hours at 37°C and heated at 90°C for 5 minutes; the antibacterial activity was then determined by a cylinder-plate method. All of the kanamycin and paromamine was inactivated.

The inactivated kanamycin was purified by chromatography on Amberlite IRC-50 and then on Dowex 1-X2. The fraction containing the inactivated kanamycin, on high-voltage paper electrophoresis, was determined with the inactivated product of tritiated kanamycin as a control.

The enzyme solution obtained from 6 liters of the cultured broth of *E. coli* ML 1629 was used to inactivate 1350 mg of kanamycin at 37°C for 20 hours. The reaction mixture contained 144 ml of tenfold-concentrated modified TMK solution, 11.5 g of adenosine triphosphate, 3.1 g of $NaHCO_3$, 1.7 g of kanamycin sulfate, and 72 ml of enzyme solution in a total volume of 240 ml. The inactivated solution was passed through a column (400 ml) of Amberlite IRC-50 and was eluted with 1 percent NH_4OH. Fractions that were ninhydrin-positive and contained a product conforming with inactivated tritiated kanamycin, according to high-voltage paper electrophoresis, were combined and further purified by chromatography on Dowex 1-X2. The crude inactivated kanamycin (300 mg) obtained in this way was further purified by Amberlite CG-50 resin chromatography, yielding two inactivated kanamycins (90 mg and 60 mg).

The 90-mg sample was reactivated by treatment with alkaline phosphatase (*2*), and the reactivation rate indicated that it contained 700 μg of kanamycin per milligram. After hydrolysis in 0.4*M* $HClO_4$ (adjusted to *p*H 4.0 with NaOH) for 20 hours at 80° to 83°C, 800 μg of kanamycin per milligram was recovered. The elementary analysis of the inactivated kanamycin showed C, 35.26; H, 6.83; N, 9.08; O, 41.76; and P, 5.20. The calculated values for $C_{18}H_{35}N_4O_{11} \cdot PO(OH)_2 \cdot 2H_2O$ are C, 36.00; H 6.88; N, 9.33; O, 42.63; and P, 5.16. Hydrolysis in 6*N* HCl at 100°C under reflux for 30 minutes showed deoxystreptamine, 3-amino-3-deoxy-D-glucose, and 6-amino-6-deoxy-D-glucose determined by thin-layer chromatography with silica gel G [*n*-propanol, pyridine, acetic acid, water (51 : 20 : 6 : 24); *n*-butanol, acetic acid, water (4 : 2 : 1)] and by high-voltage paper electrophoresis at 3500 volts for 15 minutes [acetic acid, formic acid, water (75 : 25 : 900)].

The inactivated kanamycin consumed 2.0 moles of periodate at *p*H 4.05 during 24 hours. The hydrolysis of the inactivated kanamycin after periodate oxidation gave deoxystreptamine and 6-amino-6-deoxy-D-glucose, but not 3-amino-3-deoxy-D-glucose. The consumption of 2 moles of periodate, the existence of 6-amino-6-deoxy-D-glucose, and the absence of 3-amino-3-deoxy-D-glucose in the hydrolyzate after the periodate oxidation indicate that the 3-hydroxyl group of 6-amino-6-deoxy-D-glucose moiety is phosphorylated. It conforms with the fact that this hydroxyl group is free in kanamycin, paromomycin, and neomycin to which *E. coli* ML 1629 is resistant.

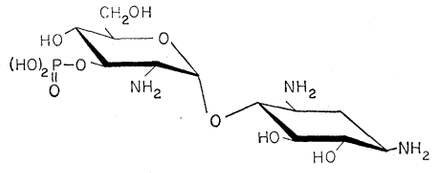


There was an unidentified product containing phosphorus in the acid hydrolyzate of the inactivated kanamycin as shown by thin-layer chromatography and high-voltage paper electrophoresis. This product was derived from the inactivated kanamycin by the secondary reaction with 6*N* HCl, because the hydrolysis with $HClO_4$ gave a theoretical amount of kanamycin.

The second inactivated product showed similar analytical values and was shown by high-voltage paper electrophoresis to be less basic than the inactivated product described above. Its conversion to kanamycin by alkaline phosphatase and phosphodiesterase (*3*) was negligible. When kanamycin was inactivated in dilute solution, such as 8 m*M* ATP and 0.7 mg of kanamycin per milliliter, the compound in which the 3-hydroxyl group of 6-amino-6-deoxy-D-glucose moiety was phosphorylated was the main inactivated compound.

Paromamine (600 mg) obtained by methanolysis of paramomycin was inactivated by the system used to inactivate kanamycin. After inactivation, the reaction mixture was passed through a column of Amberlite IRC-50 and eluted with 1 percent NH_4OH. The ninhydrin-positive eluate was lyophilized, dissolved in water, and chromatographed again on Amberlite CG-50 in the ammonium form. The inactivated paromamine appeared in the effluent with water. Concentration under reduced pressure followed by addition of methanol gave 50 mg of crystalline inactivated paromamine. The elementary anaylsis was C, 32.26; H, 6.72; N, 8.94; O, 42.24; P, 7.80. The calculated values

for $C_{12}H_{24}N_3O_7 \cdot PO(OH)_2 \cdot 2H_2O$, are C, 32.80; H, 6.88; N, 9.56; O, 43.70; P, 7.05. The crystalline inactivated paromamine consumed 1.6 moles of periodate at *p*H 4.05 in 24 hours. The hydrolysis of the periodate-oxidized inactivated paromamine gave D-glucosamine, but not deoxystreptamine. These data indicate that the 3-hydroxyl group of D-glucosamine moiety of paromamine is phosphorylated.



When the reaction mixture of inactivated kanamycin was treated by alkaline phosphatase, the recovery of kanamycin was not 100 percent; and the percentage recovery was higher when the concentrations of antibiotic and ATP in the reaction mixture were lower. When the reaction mixture containing 1 mmole of kanamycin and 8 mmole of ATP per liter was inactivated and the solution was diluted tenfold, treatment with alkaline phosphatase resulted in 60 percent recovery of kanamycin.

Adenosine triphosphate (ATP) was the absolute requirement for the inactivation reaction and addition of coenzymes (coenzyme A, nicotinamide-adenine dinucleotide, nicotinamide-adenine dinucleotide phosphate, flavin mononucleotide, flavin-adenine dinucleotide, folic acid), acetate, and amino acids did not show any enchancement of the inactivation reaction. Adenosine triphosphate could not be replaced by adenosine mono- or diphosphate, guanosine triphosphate, or uridine triphosphate.

Dihydrostreptomycin was inactivated by the kanamycin-inactivation system. Concentrations of dihydrostreptomycin and ATP in the reaction mixture were 100 μg/ml and 4 μmole/liter. After incubation at 37°C for 20 hours, the solution was heated to 90°C for 5 minutes and treated by alkaline phosphatase. This gave a 100 percent recovery of the activity. Thus, this strain of *E. coli* showed a phosphorylative inactivation of dihydrostreptomycin.

The relation between phosphorylation and resistance was shown by the fact that *E. coli* K12 and its resistant subcultures did not give an enzyme solution that inactivated kanamycin. *Escherichia coli* K12 was plated on a nutrient agar containing kanamycin, and a subculture which grew in a medium containing kanamycin (5 μg/ml) was obtained. Another resistant culture which grew in a medium containing kanamycin at 80 μg/ml was obtained by the successive transfers into a medium containing kanamycin. The enzyme solutions prepared from these resistant strains which had no R factor did not show inactivation of kanamycin.

Thus, *E. coli* that carries R factor produces an enzyme or enzymes which phosphorylate the 3-hydroxyl group of 2- or 6-amino-2- or 6-deoxy-D-glucose. Though it is not certain whether one enzyme alone catalyzes these reactions and whether the same enzyme catalyzes the same reaction on 2,6-diamino-2,6-dideoxy-D-glucose in neomycins, the isolation of the phosphorylated compounds and the dependence of the inactivation reaction on ATP indicates that phosphorylation is one of modes of inactivation of antibiotics by Gram-negative organisms carrying R factor.

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Antibody-Producing Cells in Division

Abstract. *Cells producing antibody were detected by a modified Jerne plaque technique. Of plaques initially containing a single central cell, 2.6 percent (45/1742) later had two. Mitosis of cells producing antibody was directly observed, with a mitotic time of not less than 40 minutes for the one mitosis timed at 37°C.*

A typical antibody response to a particulate antigen is characterized by the production of γM-globulin antibody followed shortly by γG-globulin. The Jerne hemolytic plaque technique (*1*) permits detection of antibody-producing cells during the γM phase of the response. The total number of these cells increases several thousandfold in the spleens of mice during a typical response. (We consistently observe that the number of antibody-producing cells doubles every 5 to 6 hours during the period 24 to 96 hours after cow red blood cells are given as antigen.) Cell division or cell recruitment, or both, could account for this increase (*2*). The experiments described here show that cells already known to be producing specific antibody do divide during the primary response, and they suggest that these cells continue to synthesize antibody after division.

We used a modified Jerne plaque technique which permits the direct observation of antibody-producing cells for many hours after they have formed hemolytic plaques (*3–7*). Plaques with central cells in good focus and with no nearby cells were selected for detailed study. In this way, it was ensured that hemolysis was the result of antibody made by central cells. Plaques, first observed after 30 minutes of incubation at 37°C, were numbered and inspected at intervals. The inspection part of the procedure was carried out at room temperature, since one experiment entirely at 37°C showed no qualitative or quantitative differences in the results at the two temperatures. Most observations were made with spleen cells taken from animals 72 hours after antigen administration, a time when the total number of antibody-producing-cells is still increasing logarithmically. Since observations made at other times (2¼ to 4 days) during the primary response do not differ importantly from these, all are described together.

When first observed, most of the plaques contained single central cells, but 4.8 percent (83/1742) already had two cells at this time. During the subsequent observation period (usually 4 to 5 hours), 2.6 percent (45/1742) of the single cells divided and gave two cells. All but one of the newly arising cell pairs remained in contact, although several of these pairs of cells showed vigorous amoeboid activity. In the instance when we did observe a