

EFFECTS OF KANAMYCIN ON PROTEIN SYNTHESIS: INHIBITION OF ELONGATION OF PEPTIDE CHAINS

JIRO SUZUKI, TAKEHIKO KUNIMOTO
and MAKOTO HORI

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

(Received for publication October 31, 1969)

Most aminoglycoside antibiotics, including streptomycin and kanamycin, not only inhibit protein synthesis but also cause misreading of the genetic code^{1,2,3,4}. The misreading was once thought to account for the lethal action of the aminoglycosides, but several observations indicate strongly that this is not the case^{5,6,7} and therefore the drugs must kill bacteria somehow through inhibition of protein synthesis. However, the precise mechanism of the inhibition remains unsolved. Recently LUZZATTO *et al.* concluded that streptomycin blocks the initiation step of protein synthesis and causes the accumulation of aberrant complex (70 S monomers)^{8,9}. On the other hand, *in vitro* experiments by MODOLELL and DAVIS showed that streptomycin inhibits chain elongation and then causes slow breakdown

of the polysomes^{10,11}.

In the present experiments we studied the correlation which might exist between the inhibitory effect of kanamycin on protein synthesis and the misreading caused by the drug, and furthermore we investigated in more detail the mechanism by which kanamycin inhibits protein synthesis both *in vivo* and *in vitro*.

If kanamycin-treated cells are forced through misreading to produce unnatural proteins which are degraded rapidly within the cells, then the inhibitory effect of kanamycin on the incorporation of radioactive amino acids would be explained by the instability of the unnatural proteins. In the experiment shown in Fig. 1, the stability of the protein which was synthesized in the presence of kanamycin was studied. *Escherichia coli* B cells were grown in nutrient broth in L-tubes (10 ml cultures) with shaking at 37°C. Kanamycin was added simultaneously with ¹⁴C-leucine. After 15 minutes the radioactive amino acid was chased by adding excess amount of unlabeled leucine. As shown in Fig. 1, the increase in the radioactivity of hot TCA insoluble fraction was diminished after chase, but there was no significant net loss of already incorporated radioactivity. Although in our experiment there is a limitation in detecting very small amount of unstable protein, the result is interpreted to show that the cells are not producing unstable proteins in such amount that would account for the reduction in

Fig. 1. Stability of the protein synthesized in the presence of kanamycin.

At zero time 20 or 5 μ g/ml of kanamycin was added in duplicate together with ¹⁴C-leucine at the final concentration of 0.5 μ Ci/ml. After 15 minutes incubation, one set of the tubes were given 10^{-2} M of ¹²C-leucine (-----). Aliquots of 4 ml were removed at 15 and 30 minutes and the radioactivity of hot TCA insoluble fractions was determined.

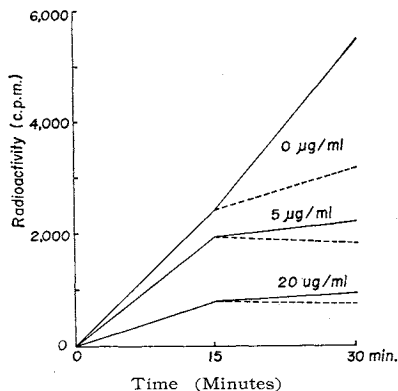


Fig. 2. Sucrose density gradient analysis of polysome profile

Polysome profiles were recorded automatically as previously described¹⁵. Sedimentation from right to left. Arrows indicate the locations of 70 S peaks.

- Butanol (0.1 M) was added 2 minutes before harvesting. Dotted line shows the profile of the normal culture.
- Kanamycin (10 μ g/ml) was added 20 minutes before harvesting.
- Kanamycin was added 20 minutes before and butanol 2 minutes before harvesting.

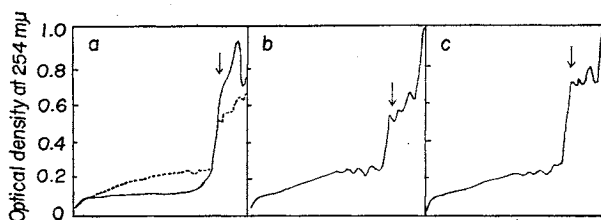


Table 1. The effect of kanamycin on puromycin reaction

Additions	c. p. m.	c. p. m. released	Per cent inhibition of release
None (before incubation)	1, 417		
None (after incubation)	1, 443		
Puromycin	1, 072	371	
Kanamycin	1, 361		
Kanamycin + puromycin	1, 154	207	44
Streptomycin	1, 406		
Streptomycin + puromycin	1, 134	272	27
Chloramphenicol	1, 439		
Chloramphenicol + puromycin	1, 193	246	34

Incubation mixtures contained ribosomes carrying ^{14}C -polyphenylalanyl tRNA (2,000 c. p. m., 1.5 O.D. unit) and antibiotics as indicated in a total volume of 0.1 ml of TMN buffer. The final concentrations of antibiotics were as follows: Puromycin, 10 $\mu\text{g}/\text{ml}$; kanamycin, 20 $\mu\text{g}/\text{ml}$; streptomycin, 20 $\mu\text{g}/\text{ml}$; chloramphenicol, 100 $\mu\text{g}/\text{ml}$. After 30 minutes of incubation at 37°C, 0.08 ml aliquots of each mixture were applied to DEAE-paper discs (25 mm in diameter). The wet discs were collected in pH 1.9 buffer (8.7% acetic acid, 2.5% formic acid) and washed by swirling. Washing was repeated as described by WEBER and DE MOSS¹⁷⁾ except that the several discs were handled in a single flask.

incorporation of C^{14} -amino acids in the presence of the inhibitor.

According to current understanding of protein synthesis, the interaction of ribosomes with mRNA can be resolved into three steps^{12,13,14)}; the formation of initiation complex, the movement of ribosomes along mRNA in concert with elongation of peptide chains and the release of ribosomes from mRNA upon the completion of protein. It is of great interest to know in which step kanamycin interrupts to stop the whole process of protein synthesis. We have recently shown that butanol, when added to intact cells, inhibits the ribosomes cycle at the initiation of protein synthesis and causes rapid dissociation of the polysome¹⁵⁾. The effect of butanol is antagonized by chloramphenicol which is a specific inhibitor of the reading out process.

In the experiments shown in Fig. 2, we checked whether kanamycin blocks the rapid dissociation of the polysome caused by butanol. *E. coli* B cells were grown in nutrient broth in L-tubes. The whole content in each L-tube was poured onto crushed ice and the lysate was prepared and analyzed by sucrose gradient centrifugation as pre-

viously described¹⁵⁾. At 2 minutes after addition of butanol, the polysomes had disappeared and ribosomes were recovered as 70 S monomers and subunits, although the discrimination of the latter are not clear under these conditions (Fig. 2a). Although the result is not shown here, the inhibition of protein synthesis by kanamycin on intact cells is manifested rather slowly. If kanamycin is added at a concentration of 10 $\mu\text{g}/\text{ml}$, it takes about 20 minutes for the drug to stop the incorporation of labeled amino acids. As shown in Fig. 2b, the polysome profile at 20 minutes after addition of 10 $\mu\text{g}/\text{ml}$ of kanamycin was not so different from that of normally growing cells, although protein synthesis at this moment had almost stopped. Furthermore, if kanamycin was added at 0 minute and butanol at 18 minutes later, the polysome profile at 20 minutes was essentially unchanged and showed little running off of ribosomes from mRNA (Fig. 2c). Since butanol blocks the initiation step of protein synthesis, it is evident that kanamycin somehow freezes the polysome and thus prevents the ribosomes from moving along mRNA.

Another evidence supporting the above conclusion was obtained by studying the effect of kanamycin on the puromycin-dependent release of nascent peptide chains *in vitro*. S-30 fraction was prepared from *E. coli* Q 13 cells and mixed with conventional components for poly U-dependent ^{14}C -phenylalanine incorporation as described by MATTHAEI and NIRENBERG¹⁶⁾. After incubation for 25 minutes at 37°C, the reaction mixture was diluted with TMN buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M Mg acetate and 0.16 M NH_4Cl) and centrifuged for 45 minutes at 180,000 g to collect the ribosomes carrying labeled peptidyl tRNA. The ribosomes were resuspended in TMN buffer and again centrifuged for 10 minutes at 5,000 g to remove aggregated material. The ribosome suspension was diluted with TMN buffer and incubated with puromycin or puromycin plus other antibiotics. The release of peptides from tRNA was measured by applying aliquots of incubation mixtures to DEAE-paper discs as described by WEBER and DE MOSS¹⁷⁾. The results are shown in Table

1. Chloramphenicol inhibited the puromycin reaction as described by several investigators^{17,18,19}. The inhibitory effect of kanamycin on the puromycin reaction was also clearly demonstrated.

Since the puromycin reaction can be considered as a model of the elongation of peptide chains, it is concluded that kanamycin somehow inhibits the elongation of peptide chains and prevents the ribosomes from moving along mRNA. As shown in Table 1, kanamycin inhibited the puromycin reaction more markedly than streptomycin did. While, as reported by CERNA *et al.*²⁰, streptomycin is an inhibitor of the reaction, MODOLELL and DAVIS claim that it does not inhibit the reaction¹¹. It remains to be determined whether the discrepancy depends on the conditions.

Furthermore, as mentioned earlier, there is a controversy as to which step of ribosome cycle streptomycin blocks. The results we obtained seem to permit us to conclude that kanamycin inhibits the elongation of peptide chains, in which a series of reactions are involved, resulting in preventing ribosomes from moving along mRNA. So far as this mechanism of action is concerned, our conclusion does not conflict with that of MODOLELL and DAVIS¹¹.

Acknowledgement

We wish to thank Prof. HAMA O UMEZAWA of this Institute for his interest in this work and helpful discussion.

References

- 1) FLAKS, J. G.; E. C. COX & J. R. WHITE : Inhibition of polypeptide synthesis by streptomycin. *Biochem. Biophys. Res. Commun.* 7 : 385~389, 1962
- 2) SPEYER, J. F.; P. LENGYEL & C. BASILIO : Ribosomal localization of streptomycin sensitivity. *Proc. Natl. Acad. Sci. U. S.* 48 : 684~686, 1962
- 3) DAVIES, J.; W. GILBERT & L. GORINI : Streptomycin, suppression and the code. *Proc. Natl. Acad. Sci. U. S.* 51 : 883~890, 1964
- 4) GORINI, L. & E. KATAJA : Phenotypic repair by streptomycin of defective genotypes in *E. coli*. *Proc. Natl. Acad. Sci. U. S.* 51 : 487~493, 1964
- 5) WHITE, J. R. & H. L. WHITE : Streptomycin-oid antibiotics : Synergism by puromycin. *Science* 146 : 772~774, 1964
- 6) GORINI, L. & E. KATAJA : Streptomycin-induced oversuppression in *E. coli*. *Proc. Natl. Acad. Sci. U. S.* 51 : 995~1001, 1964
- 7) BROWNSTEIN, B. L. & L. J. LEWANDOWSKI : A mutation suppressing streptomycin dependence. I. An effect on ribosome function. *J. Mol. Biol.* 25 : 99~109, 1967
- 8) LUZZATTO, L.; D. APIRION & D. SCHLESSINGER : Mechanism of action of streptomycin in *E. coli* : Interruption of the ribosome cycle at the initiation of protein synthesis. *Proc. Natl. Acad. Sci. U. S.* 60 : 873~880, 1968
- 9) LUZZATTO, L.; D. APIRION & D. SCHIESSINGER : Polyribosome depletion and blockage of the ribosome cycle by streptomycin in *E. coli*. *J. Mol. Biol.* 42 : 315~335, 1969
- 10) MODOLELL, J. & B. D. DAVIS : Rapid inhibition of polypeptide chain extension by streptomycin. *Proc. Natl. Acad. Sci. U. S.* 61 : 1279~1286, 1968
- 11) MODOLELL, J. & B. D. DAVIS : Mechanism of inhibition of ribosomes by streptomycin. *Nature* 224 : 345~348, 1969
- 12) MANGIAROTTI, G. & D. SCHLESSINGER : Polysome metabolism in *E. coli*. II. Formation and lifetime of messenger RNA molecules, ribosomal subunit couples and polysomes. *J. Mol. Biol.* 29 : 395~418, 1967
- 13) KAEMPFFER, R. O. R.; M. MESELSON & H. J. RASKAS : Cyclic dissociation into stable subunits and re-formation of ribosomes during bacterial growth. *J. Mol. Biol.* 31 : 277~289, 1968
- 14) NOMURA, M. & C. V. LOWRY : Phage f2 RNA-directed binding of formylmethionyl-tRNA to ribosomes and the role of 30 S ribosomal subunits in initiation of protein synthesis. *Proc. Natl. Acad. Sci. U. S.* 58 : 946~953, 1967
- 15) HORI, M.; T. KUNIMOTO & J. SUZUKI : Rapidly reversible dissociation of polysomes in *E. coli* by *n*-butanol. *J. Biochem.* 66 : 705~709, 1969
- 16) MATTHAEI, J.H. & M.W. NIRENBERG : Characteristics and stabilization of DNase-sensitive protein synthesis in *E. coli* extracts. *Proc. Natl. Acad. Sci. U. S.* 47 : 1580~1588, 1961
- 17) WEBER, M. J. & J. A. DE MOSS : Inhibition of the peptide bond synthesizing cycle by chloramphenicol. *J. Bact.* 97 : 1099~1105, 1969
- 18) MONRO, R. E. & K. A. MARCKER : Ribosome-catalysed reaction of puromycin with a formylmethionine-containing oligonucleotide. *J. Mol. Biol.* 25 : 347~350, 1967
- 19) CANNON, M. : The puromycin reaction and its inhibition by chloramphenicol. *European J. Biochem.* 7 : 137~145, 1968
- 20) CERNA, J.; I. RYCHLIK & P. PULKRABEK : The effect of antibiotics on the coded binding of peptidyl-tRNA to the ribosomes and on the transfer of the peptidyl residue to puromycin. *European J. Biochem.* 9 : 27~35, 1969