Studies on the Mechanism of Action of Gentamicin. Effect on Protein Synthesis in Cell-Free Extracts of *Escherichia coli**

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ABSTRACT: Addition of Gentamicin to exponentially growing cells of *Escherichia coli* strongly inhibits amino acid incorporation into protein while uracil incorporation into nucleic acid is not affected. When the antibiotic is added to cell-free extracts of *E. coli* in the presence of natural (homologous or heterologous) messenger ribonucleic acids (m-RNA's), it stimulates consistently the incorporation of many amino acids. In the presence of polyuridylic acid (poly U), Gentamicin induces a "misreading" effect by stimulating incorporation of isoleucine. In cell-free extracts prepared from a mutant resistant to 20 μg/ml of Gentamicin, the incorporation of amino acids directed by endogenous m-RNA's is not stimulated even by 100 μg/ml of Gentamicin.

The resistance to the stimulation of amino acids incorporation appears to be a property of a fraction of the 105,000*g* supernatant. A "misreading" effect is evident also in this mutant, even in the absence of the antibiotic.

Gentamicin is an aminoglycosidic antibiotic produced by species of *Micromonospora* which is active against gram-positive and gram-negative bacteria (Weinstein *et al.*, 1963a,b). Davies and co-workers (1965b) have shown that it causes a strong "misreading" effect when protein synthesis by cell-free extracts from *Escherichia coli* is directed by synthetic polyribonucleotides.

The purpose of this investigation was to study the effect of Gentamicin on protein synthesis *in vivo* and *in vitro*. Furthermore, an attempt has been made to localize the cellular site which is sensitive to the action of the antibiotic, as revealed by its effect on amino acid incorporation by cell-free extracts.

Materials and Methods

L-[^{14}C]Amino acids of high specific activity (from 32 to 367 μc/μmole), L-[*methyl*-^{14}C]methionine (13.4 μc/μmole), and [2-^{14}C]uracil (8.6 μc/μmole) were purchased either from New England Nuclear Corp. or from Volk Radiochemical Co. Synthetic polynucleotides were obtained from Miles Chemical Co. Gentamicin was kindly supplied by Schering Corp. Tobacco mosaic virus (TMV) was a generous gift of Dr. E. Baldacci of the Institute of Plant Pathology, University of Milan.

Bacterial Strains. E. coli K 12 and *E. coli* 746 (a K 12 derivative requiring histidine, methionine, and thymine for growth) were used as Gentamicin-sensitive strains. Spontaneous mutants resistant to Gentamicin were selected on solid medium Penassay (Difco) and their level of resistance was evaluated in both liquid and solid media. The frequency of resistant mutants was about 10^{-8}. Mutants resistant to more than 20 μg/ml were not obtained as a result of single-step mutations. All of these mutants proved to be very unstable and were easily lost even when stored as slant cultures at 3–5°.

Cell-Free Protein Synthesis. Cells were grown aerobically at 37° in Penassay broth (Difco), harvested in logarithmic growth phase, and washed in the standard buffer of Nirenberg and Matthaei (1961).

Ribosomes and enzyme fractions were prepared by methods similar to those reported by Wood and Berg (1962). After grinding with alumina (Alcoa A-305) and addition of 3 ml of standard buffer/g of cells, the suspension was centrifuged at 12,000*g* for 20 min and then at 20,000*g* for 30 min. Ammonium sulfate (1.4 g/10 ml of extract) was added and the precipitate was discarded by centrifugation for 15 min at 12,000*g*. The supernatant was dialyzed against standard buffer for 8 hr at 4°. An aliquot of the preparation was treated for 10 min at 4° with 2.5 μg/ml of deoxyribonuclease (DNase) (Worthington Biochemical Corp.) and stored at −20° (S-20 fraction).

The remainder of the preparation was centrifuged at 105,000*g* for 3 hr. The ribosomal pellet was resuspended in 0.01 M Tris–HCl, pH 7.8–3 M KCl–0.04 M magnesium acetate and washed by differential centrifugation. The ribosomal pellet was resuspended in standard buffer to give a final concentration of 30–40 mg/ml of ribosomal protein, dialyzed overnight at 4° against standard buffer, and stored at −20°.

The upper four-fifths of the supernatant from the first centrifugation at 105,000*g* was removed with a pipet and an aliquot was treated with protamine sulfate (Mann Research Laboratory) (0.2 ml of 1%

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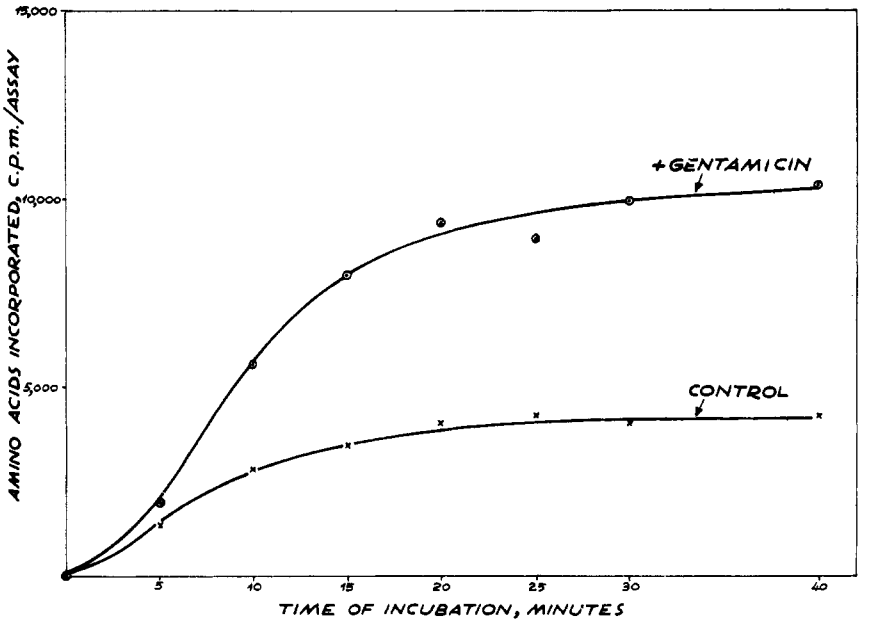

FIGURE 1: Time course of amino acid incorporation directed by TMV–RNA in the presence and in the absence of Gentamicin. Composition of reaction mixtures as in Table II, except 0.1 μc each of L-[^{14}C]lysine, L-[^{14}C]arginine, L-[^{14}C]leucine, and L-[^{14}C]isoleucine were added. When present, 100 μg/ml of Gentamicin was added.

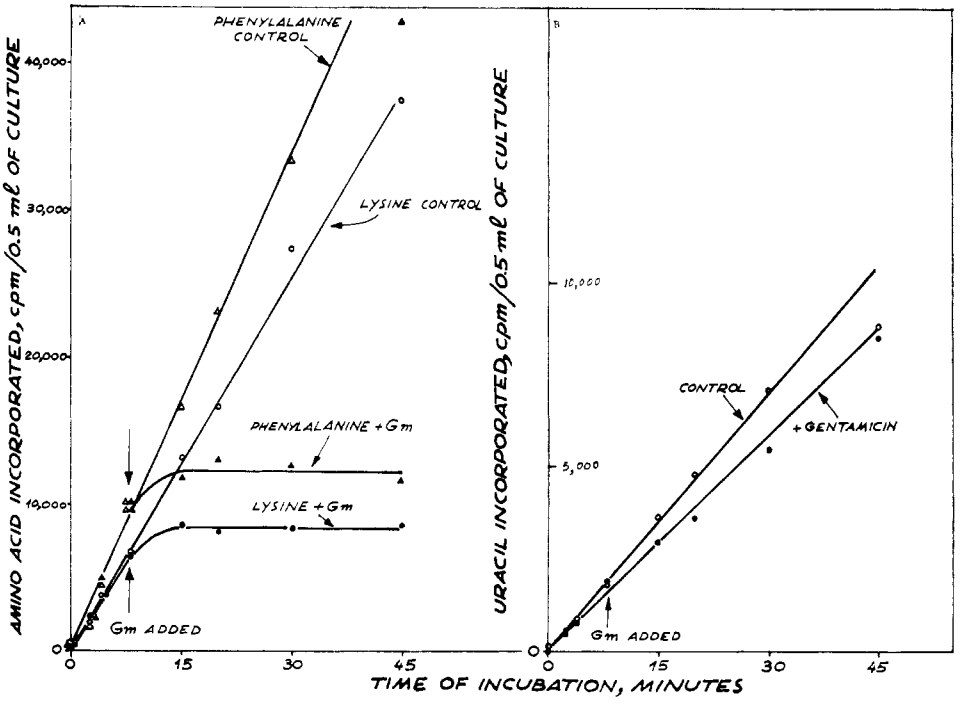

FIGURE 2: Effect of Gentamicin on the incorporation of amino acids and uracil by growing cells of *E. coli*. Cells grown overnight on Penassay medium were harvested by centrifugation, washed once with saline solution, and suspended in Davis minimal medium (Davis and Mingioli, 1950). Aliquots of this suspension (4.8×10^7 cells) were used to inoculate flasks containing 10 ml each of Davis minimal medium. The flasks were kept at 37° on a reciprocate shaker for 3 hr. After that time, 10 μc of L-[^{14}C]lysine, L-[^{14}C]phenylalanine, or [2-^{14}C]uracil was added to duplicate flasks. After 8 min of incubation, Gentamicin to give a final concentration of 50 μg/ml was added to a group of flasks. Samples (0.5 ml) of cell culture were removed at the indicated time and added to 2 ml of either 10% trichloroacetic acid (in the case of the amino acids) or 0.8 M perchloric acid (in the case of uracil). After standing in the cold for 60 min, the samples were filtered on Millipore filters, washed with either 5% trichloroacetic acid or 0.2 M perchloric acid, dried, and counted.

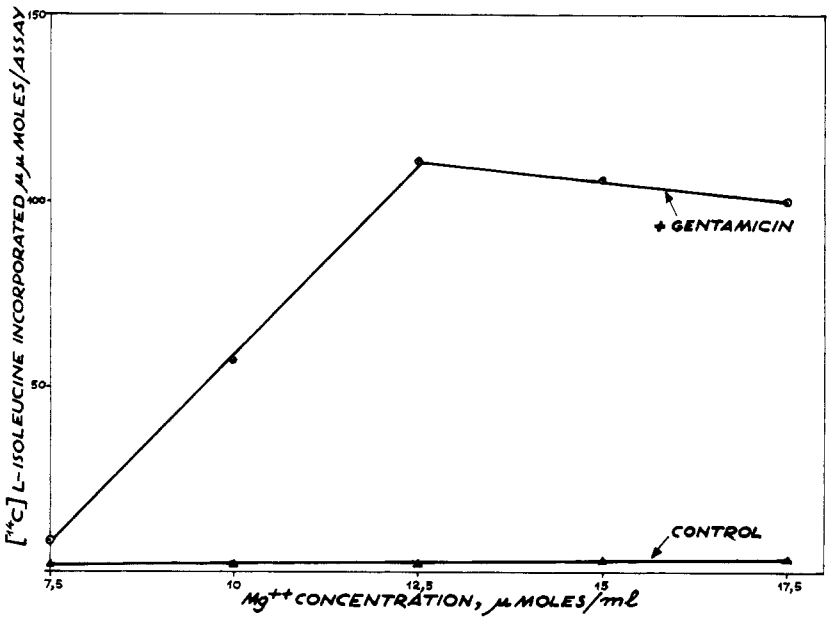

FIGURE 3: Influence of magnesium concentration on Gentamicin-induced "miscode." The reaction mixtures (0.25 ml) contained (micromoles per milliliter) Tris-HCl (pH 7.8), 50; NH_4Cl, 75; Mg^{2+} as indicated; mercaptoethanol, 10; GTP, 0.2; ATP, 2; and PEP, 5; pyruvate kinase, 25 μg/ml; 19 [^{12}C]amino acids (except isoleucine), 5×10^{-2} each; and 0.2 μc of L-[^{14}C]isoleucine. In addition, the reaction mixture contained 1.4 mg of ribosomes, supernatant enzymes corresponding to 200 μg of proteins, 200 μg of s-RNA, and 20 μg of poly U. Gentamicin (10 μg/ml) was added where indicated. Samples were preincubated for 30 min at 37° in the absence of L-[^{14}C]isoleucine, poly U, and Gentamicin; incubation was then carried out for 45 min.

protamine solution/ml of supernatant). After standing at 0° for 15 min, the precipitate was removed by centrifugation at 12,000*g* for 15 min and the supernatant was stored at −20° (supernatant enzymes).

An aliquot of the supernatant after the first 105,000*g* centrifugation was treated for 15 min at room temperature with 2.5 μg/ml of DNase and the soluble ribonucleic acid (s-RNA) was extracted with phenol according to the method of Nirenberg and Matthaei (1961). The s-RNA precipitate was dissolved in 0.01 M Tris-HCl, pH 7.8, and dialyzed against the same buffer overnight at 4°.

Assays for protein synthesis were performed according to the method of Nirenberg and Matthaei (1961). The composition of the reaction mixtures and the details of the assays are specified in the legends of the figures and tables. After incubation, the reactions were stopped by adding four volumes of 10% trichloroacetic acid containing 1% casamino acids (Difco); samples were then immersed in boiling water for 15 min, kept at 4° for at least 30 min, filtered on filter paper (Schleicher and Schull no. 5893), and washed on the filter with 10 ml of 5% trichloroacetic acid containing 1.5% casamino acids, followed by ethanol–ether and ether. Counting was performed on either a gas-flow counter (counting efficiency 17.5%) or in a liquid scintillation counter (counting efficiency 35%).

Preparation of TMV-RNA. RNA was extracted from a suspension of tobacco mosaic virus by the phenol procedure and precipitated from the aqueous layer with cold ethanol. The precipitate was recovered by centrifugation, dissolved in 0.01 M Tris-HCl, pH 7.8, and dialyzed at 4° against the same buffer.

Preparation of L-[^{14}C]Phenylalanyl-s-RNA. The reaction mixture for the preparation of L-[^{14}C]phenylalanyl-s-RNA contained (in micromoles per milliliter): Tris-HCl (pH 7.8), 50; NH_4Cl, 75; magnesium acetate, 10; mercaptoethanol, 10; ATP,[1] 5; and PEP, 5; pyruvate kinase, 25 μg/ml; 19 [^{12}C]amino acids (except phenylalanine), 5×10^{-2} each; L-[^{14}C]phenylalanine (sp act. 367 μc/μmole), 0.6 μc; approximately 2 mg/ml of s-RNA; and 1 mg/ml of supernatant enzymes. After incubation for 20 min at 37°, the reaction was stopped by adding one volume of cold, water-saturated phenol and s-RNA was extracted and purified as reported by Kaji and co-workers (1965).

Binding of Phenylalanyl-s-RNA to Ribosomes. Binding of L-[^{14}C]phenylalanyl-s-RNA to ribosomes was measured by a slight modification of the method reported by Nirenberg and Leder (1964). The 0.25-ml reaction mixtures contained (micromoles per milliliter): Tris-HCl (pH 7.2), 100; NH_4Cl, 50; magnesium

[1] Abbreviations used: ATP and GTP, adenosine and guanosine triphosphates; PEP, phosphoenolpyruvate.

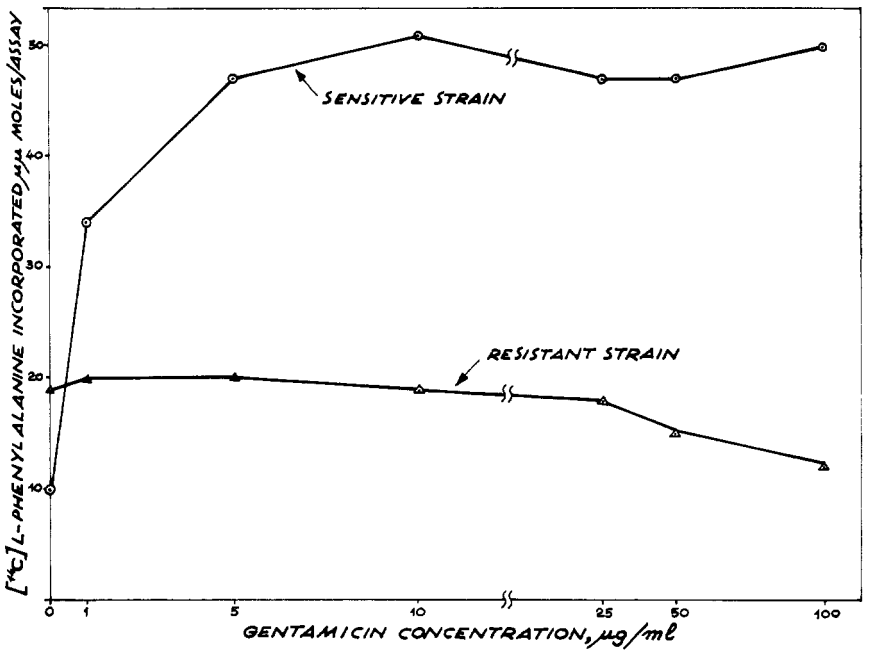

FIGURE 4: Effect of Gentamicin concentration on the incorporation of L-[^{14}C]phenylalanine by extracts from the sensitive and the resistant strains. Reaction mixtures as in Figure 3 except that Mg^{2+} concentration was 10^{-2} M, and 0.3 μc of L-[^{14}C]phenylalanine was added per assay; S-20 fraction corresponding to 1 mg of protein and 200 μg of RNA were used in the case of both Gentamicin-sensitive and -resistant strains. Incubation was for 45 min.

acetate, 60; mercaptoethanol, 10; GTP, 0.2; and PEP, 5; pyruvate kinase, 25 μg/ml; polyuridylic acid (poly U), 80 μg/ml; and puromycin, 80 μg/ml. Ribosomes were preincubated for 30 min at 37° in the presence of 40 μg/ml of puromycin, ATP and an ATP-regenerating system, and 20 unlabeled amino acids. After preincubation, the solution was diluted with cold standard buffer, centrifuged at 105,000*g* for 2 hr, and resuspended in standard buffer. Where indicated, supernatant enzymes and Gentamicin (10 μg/ml) were added. Incubation was carried out at 24° for 20 min, then 5 ml of cold 0.1 M Tris, pH 7.2–0.02 M magnesium acetate–0.05 M KCl was added. Aliquots (1 ml) were removed, and polyphenylalanine synthesis was measured by the technique already reported except that Millipore filters were used and washing with organic solvents was omitted. The remainder of the diluted reaction mixtures was filtered on Millipore filters and washed on the filters as described by Nirenberg and Leder (1964). The filters were placed into liquid scintillation glass vials, air dried, and counted in a Packard counter.

Results

Effect of Gentamicin on the Incorporation of Amino Acids into Protein by Cell-Free Extracts in the Presence of Natural m-RNA. The effect of Gentamicin (100 μg/ml) on the incorporation of 13 amino acids as directed by endogenous m-RNA's is shown in Table I. The antibiotic stimulated the incorporation into protein of 10 of the 13 amino acids tested, while proline, glycine, and valine were not affected. The remaining amino acids were either not tested or they gave control values too high to provide significant differences on the extent of incorporation. In no case, however, was inhibition of the incorporation observed.

Gentamicin also caused a tenfold stimulation in the incorporation of L-[^{14}C]lysine and [^{14}C]glycine into protein when RNA extracted from tobacco mosaic virus was employed as a messenger in place of the natural endogenous m-RNA (Table II). Appropriate controls demonstrate that the antibiotic acts only under conditions that allow cell-free protein synthesis.

In the presence of Gentamicin, the stimulation of amino acid incorporation was evident already in the early minutes of incubation and a plateau was reached at approximately the same time as in the absence of Gentamicin (Figure 1). Attempts to observe a stimulation of amino acids incorporation *in vivo* were unsuccessful insofar as the addition of Gentamicin to exponen-

TABLE I: Effect of Gentamicin on the Incorporation of Amino Acids Directed by Endogenous m-RNA.[a]

Amino Acid	μμmoles Incorporated — Gentamicin	μμmoles Incorporated + Gentamicin	Δ (μμmoles)	Stimulation, -Fold
Histidine	8.8	28.9	20.1	3.28
Lysine	17.3	51.4	34.1	2.97
Tyrosine	10.6	29.1	18.5	2.74
Phenylalanine	16.1	36.4	20.3	2.26
Isoleucine	26.5	54.1	27.6	2.04
Arginine	19.2	37.3	18.1	1.94
Alanine	47.5	78.0	30.5	1.64
Glutamic acid	12.3	20.1	7.8	1.63
Serine	26.0	40.8	14.8	1.56
Tryptophan	67.5	78.0	10.5	1.15
Valine	20.9	20.6	−0.3	—
Glycine	27.3	26.3	−1.0	—
Proline	19.8	18.4	−1.4	—

[a] Reaction mixtures (final volume, 0.25 ml) contained the following components (micromoles per milliliter): Tris-HCl (pH 7.8), 50; NH_4Cl, 75; magnesium acetate, 10; mercaptoethanol, 10; GTP, 0.2; ATP, 3; and PEP, 5; pyruvate kinase, 25 μg/ml; 19 [^{12}C]amino acids except the labeled one 5×10^{-2} each; 0.3 μc/assay of radioactive amino acid; and S-20 fraction from *E. coli* K 12 corresponding to 1.2 mg of protein (see Materials and Methods). Where indicated 25 μg of Gentamicin was added. Samples were incubated for 45 min at 37°.

TABLE II: Effect of Gentamicin on the Incorporation of L-[^{14}C]Lysine and [^{14}C]Glycine Directed by TMV-RNA.[a]

	Cpm/Assay
Complete	438
+Gentamicin	4327
−TMV-RNA	114
−TMV-RNA + Gentamicin	652
−ATP, PEP, and pyruvate kinase	39
−ATP, PEP, pyruvate kinase + Gentamicin	59
+RNase + Gentamicin	41
Complete incubated at 0° + Gentamicin	50
Complete deproteinized at zero time	51
−TMV-RNA, nonpreincubated	1843
−TMV-RNA, nonpreincubated + Gentamicin	4818

[a] Conditions as in Table I except that [^{12}C]lysine and [^{12}C]glycine were omitted from the amino acids mixture and 0.2 μc of L-[^{14}C]lysine and [^{14}C]glycine were present in each assay. When present, 170 μg of TMV-RNA, 5 μg of RNase, and 25 μg of Gentamicin were added to each reaction mixture. Unless otherwise indicated, samples were preincubated for 30 min at 37° in the absence of L-[^{14}C]lysine, [^{14}C]glycine, TMV-RNA, and Gentamicin. Incubation was then carried out for 45 min.

tially growing cultures of *E. coli* caused a sudden arrest in the incorporation of amino acids into protein but not of uracil into ribonucleic acids (Figure 2).

Effect of Gentamicin on the Incorporation of Amino Acids into Protein by Cell-Free Extracts in the Presence of Poly U. Addition of Gentamicin (10 μg/ml) to cell-free extracts of *E. coli* stimulated the incorporation of isoleucine in the presence of poly U ("miscode"), as reported by Davies *et al.* (1965b). The optimal Mg^{2+} concentration for this effect is 12.5 μmoles/ml as shown in Figure 3.

It is evident, therefore, that Gentamicin interferes with protein synthesis by cell-free extracts from *E. coli* when the polypeptide synthesis is directed either by natural m-RNA's or by synthetic polyribonucleotides. In the former case, Gentamicin appears to cause a general stimulation of amino acid incorporation, whereas in the latter it strongly stimulates the incorporation of amino acids which normally are not coded for by the synthetic polyribonucleotides used, as previously reported by Davies *et al.* (1965a).

The experiments reported below show that these two effects may be separated by comparing the properties of the cell-free systems obtained from Gentamicin-sensitive and -resistant strains. Furthermore, it will be shown that the stimulatory effect in the presence of natural m-RNA's seems to be more representative of the mode of action of the antibiotic, even though both effects may be due to the same mechanism of interaction of the antibiotic with the protein-synthesizing system.

Incorporation of Amino Acids into Protein by Cell-Free Extracts Prepared from a Mutant of E. coli Resistant to Gentamicin. Figure 4 reports the dose response curves for the incorporation of phenylalanine directed by endogenous m-RNA's by cell-free extracts prepared from the sensitive strain and from a strain resistant to 20 μg/ml of Gentamicin. (Values obtained at zero concentration of antibiotic show that the preparations from both strains had approximately the same activity.) In the presence of the antibiotic, the system prepared from the resistant strain is not stimulated even at high concentrations of Gentamicin while the system prepared from the sensitive strain is stimulated from three- to fivefold. Similar results were obtained with isoleucine (Figure 5) and when a reconstructed system using ribosomes, supernatant enzymes, and s-RNA (see Materials and Methods) was employed in place of the "S-20" fraction. Phenylalanine–isoleucine "miscode" induced by Gentamicin

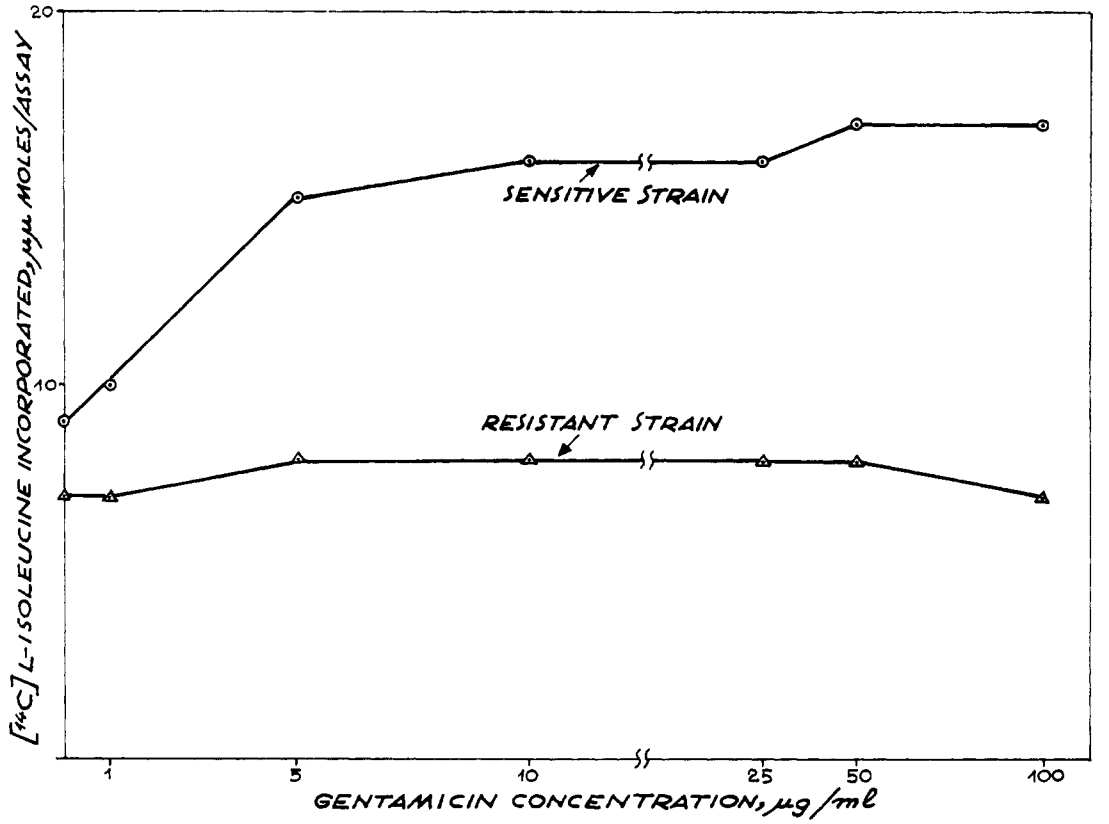

FIGURE 5: Effect of Gentamicin concentration on the incorporation of L-[^{14}C]isoleucine by extracts from the sensitive and the resistant strains. Experimental conditions as in Figure 4, except that isoleucine was omitted from the mixture of 19 [^{12}C]amino acids and 0.3 μc/assay of L-[^{14}C]isoleucine was used.

TABLE III: Effect of Gentamicin on the Incorporation of L-[^{14}C]Phenylalanine by Homologous and Heterologous Systems from the Sensitive and the Resistant Strains.[a]

Ribosomes	Supernatant Enzymes	s-RNA	L-[^{14}C]Phenylalanine Incorp (μμmoles/assay)		
			−Gentamicin	+Gentamicin	Δ
Sensitive	Sensitive	Sensitive	0.8	16.1	15.3
Sensitive	Sensitive	Resistant	5.7	22.4	16.7
Sensitive	Resistant	Sensitive	1.7	4.3	2.6
Sensitive	Resistant	Resistant	2.7	4.4	1.7
Resistant	Resistant	Resistant	3.7	2.9	0
Resistant	Resistant	Sensitive	2.5	1.4	0
Resistant	Sensitive	Resistant	5.3	15.3	10.0
Resistant	Sensitive	Sensitive	3.7	16.2	12.5

[a] Conditions as in Table I, except that the S-20 fraction was substituted by 2.6 mg of ribosomes, supernatant enzymes corresponding to 200 μg of protein, and 200 μg of s-RNA from either the resistant or the sensitive strain.

in the cell-free systems from both strains is illustrated in Figures 6 and 7.

In both cases a strong incorporation of isoleucine in the presence of poly U was noticed when Gentamicin was present and the effect increased in magnitude by increasing the concentration of s-RNA (Figure 6a and b). A marked quantitative difference was observed in the extent of stimulation in the two strains. Thus, at the highest s-RNA concentration the incorporation of isoleucine by the extract from the sensitive strain was about 100-fold above the control containing no antibiotic, whereas in the extract from the resistant

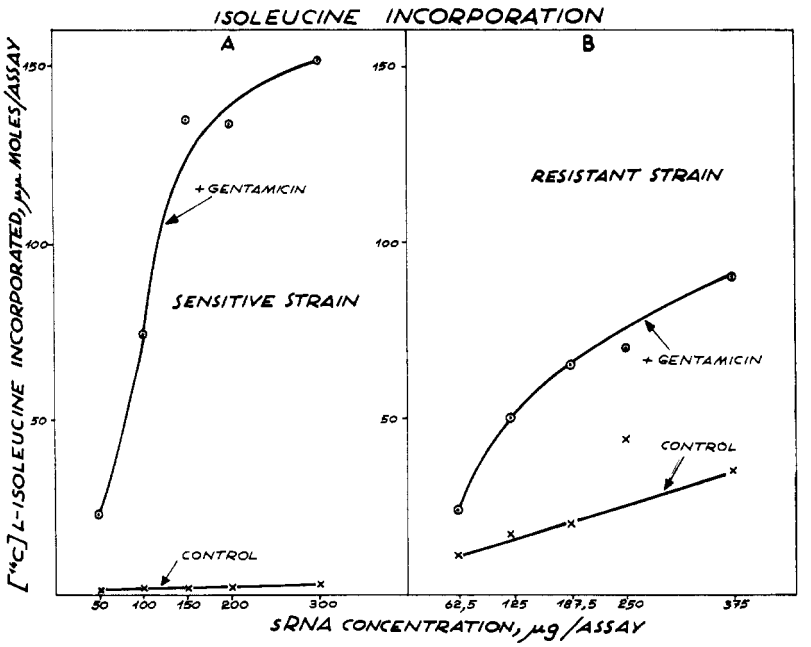

FIGURE 6: Influence of Gentamicin on the incorporation of L-[^{14}C]isoleucine directed by poly U in the cell-free systems prepared from sensitive and resistant strains. Reaction mixtures (0.25 ml) had the following composition (in micromoles per milliliter): Tris–HCl (pH 7.8), 50; NH_4Cl, 75; magnesium acetate, 12.5; mercaptoethanol, 10; GTP, 0.2; ATP, 3; and PEP, 5; pyruvate kinase, 25 μg/ml; 19 [^{12}C]amino acids (isoleucine omitted), 5 × 10^{-2} each; and L-[^{14}C]-isoleucine, 0.2 μc. In addition 1.2 mg of ribosomes, supernatant enzymes corresponding to 250 μg of protein, 20 μg of poly U, and 10 μg/ml of Gentamicin were added where indicated. Samples were preincubated for 30 min in the absence of poly U and Gentamicin; incubation was then carried out for 45 min; (a) sensitive strain, (b) resistant strain.

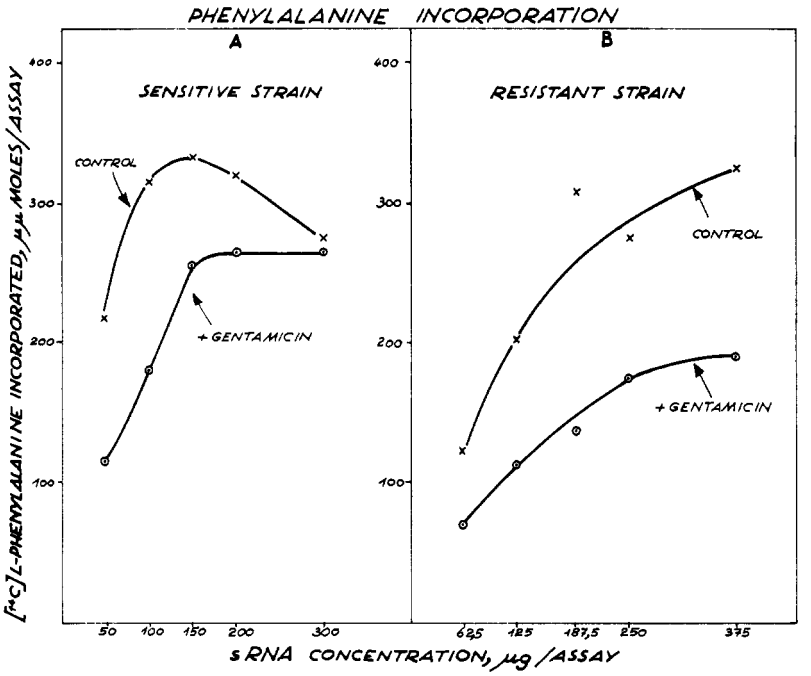

FIGURE 7: Influence of Gentamicin on the incorporation of L-[^{14}C]phenylalanine directed by poly U in the cell-free systems prepared from sensitive and resistant strains. Reaction mixtures as in Figure 6, except that L-[^{14}C]phenylalanine was used in place of L-[^{14}C]isoleucine and that the 19 [^{12}C]amino acids contained isoleucine in place of phenylalanine; (a) sensitive strain, (b) resistant strain.

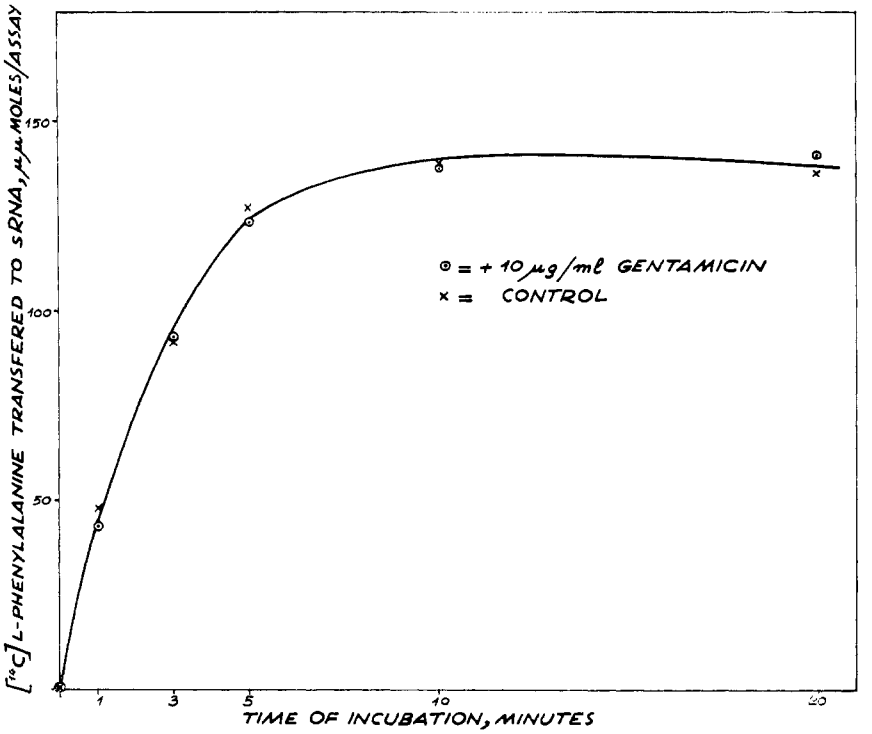

FIGURE 8: Influence of Gentamicin on the transfer of L-[^{14}C]phenylalanine to s-RNA. Composition of the reaction mixtures (0.25 ml) as described for the preparation of L-[^{14}C]phenylalanyl-s-RNA (see Materials and Methods). Gentamicin (10 μg/ml) was added where indicated. Reactions were stopped at the indicated times with 1 ml of cold, 0.8 M perchloric acid, kept at 4° for 30 min, filtered, the precipitate was washed on the filter with 10 ml of cold, 0.2 M perchloric acid, ethanol–ether, and ether, and counted.

strain it was at the most threefold. However, it can clearly be seen that an effect of misreading is quite evident in the extract from resistant strain even in the absence of the antibiotic (Figure 6b).

The effect of Gentamicin on the incorporation of phenylalanine directed by poly U is also dependent on s-RNA concentration (Figure 7). In the system prepared from the resistant strain Gentamicin always inhibits phenylalanine incorporation regardless of s-RNA concentration (Figure 7b), whereas in the system from the sensitive strain the inhibitory effect is practically negligible at the highest concentrations of s-RNA (Figure 7a).

Attempts to Localize the Cellular Fraction Sensitive to the Action of Gentamicin. Table III reports the effect of Gentamicin on the incorporation of amino acids in the systems prepared by interchanging the components (ribosomes, supernatant enzymes, and s-RNA) obtained from the resistant and the sensitive strains. It appears that the supernatant enzymes are responsible for the resistance to the stimulation brought about by Gentamicin on the incorporation of amino acids directed by endogenous m-RNA's. In fact, the stimulatory effect of the antibiotic was abolished when the enzyme fraction from the resistant strain was used regardless of the source of the ribosomes and s-RNA. Conversely, if the supernatant enzymes from the sensitive strain were employed, stimulation of amino acid incorporation always occurred, even in the presence of ribosomes and s-RNA from the resistant strain.

Since no appreciable degradation or inactivation of the antibiotic, as measured by the residual antibiotic activity, was observed when Gentamicin was incubated with the supernatant enzymes prepared from the resistant strain, it may be surmised that resistance to the antibiotic may be associated with the enzymes involved in protein synthesis. Further attempts to determine which of the reactions dependent on the supernatant enzymes is responsible for the stimulatory action of Gentamicin provided only negative evidence. Figure 8 shows that in the preparation obtained from the sensitive strain, Gentamicin has no effect on the transfer of phenylalanine to s-RNA.

Figure 9 reports the results of an experiment in which the effect of Gentamicin on the binding of phenylalanyl-s-RNA to ribosomes, directed by poly U, was studied in the presence or in the absence of the supernatant enzymes. All the fractions were obtained from the sensitive strain, and L-[^{14}C]phenylalanyl-s-RNA was added in excess to ribosomes. To avoid synthesis of polyphenylalanine, a Mg^{2+} concentration higher than that required for protein synthesis and 80 μg/ml of puromycin were employed. Under these conditions, protein synthesis is strongly inhibited but no effect is evident on the binding of

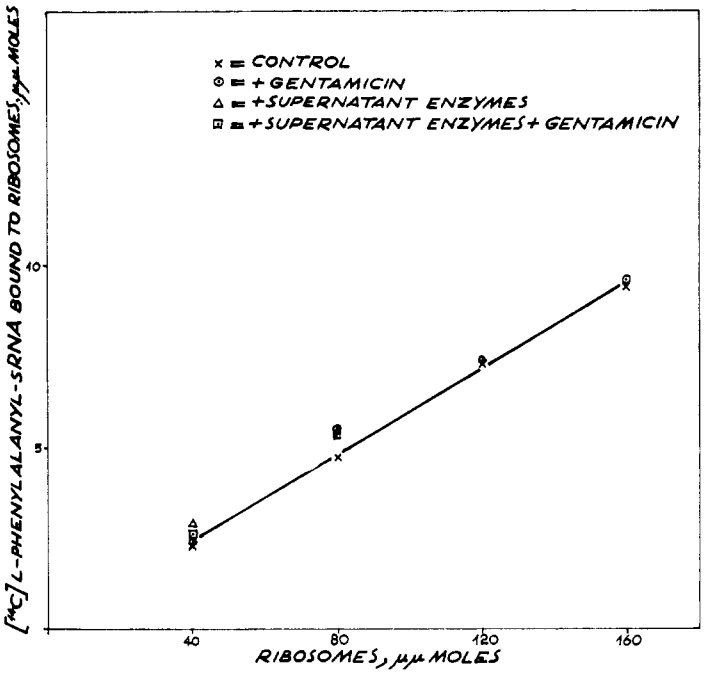

FIGURE 9: Influence of Gentamicin on the poly U directed binding of phenylalanyl-s-RNA to ribosomes. Each reaction mixture contained 60 $\mu\mu$moles of L-[^{14}C]phenylalanine bound to s-RNA (approximately 750 $\mu\mu$moles of s-RNA) and limiting amounts of ribosomes preincubated with puromycin. Where indicated, supernatant enzymes corresponding to 200 μg of protein and 10 μg/ml of Gentamicin were added. Controls for polyphenylalanine synthesis gave values corresponding to less than 0.1 $\mu\mu$mole/assay. The molecular weight of s-RNA and 70-S ribosome was assumed to be 2.7×10^4 and 2.7×10^6, respectively.

aminoacyl-s-RNA to ribosomes, as already reported by other workers (Seeds and Conway, 1966) and as confirmed in our laboratory in control experiments. It can be seen that Gentamicin does not affect the extent of binding, neither in the presence nor in the absence of the supernatant enzymes. Since the ribosomes concentration is limiting under these conditions, it can be concluded that Gentamicin does not increase the number of ribosomes available for the binding with phenylalanyl-s-RNA.

Similar experiments, not reported here, showed that Gentamicin does not increase the number of aminoacyl-s-RNA which can bind to ribosomes, when the aminoacyl-s-RNA concentration was the limiting factor, and that Gentamicin does not significantly affect the rate of binding of aminoacyl-s-RNA to ribosomes. It has to be noted, however, that in all these experiments the binding of aminoacyl-s-RNA was independent of the addition of the supernatant enzymes (Conway, 1964).

Discussion

Aminoglycosidic antibiotics are known to affect protein synthesis in bacteria. Experiments performed with growing cells showed an immediate arrest of the uptake of amino acids from the culture medium and their incorporation into proteins in the case of streptomycin (Anand and Davis, 1960), spectinomycin (Davies *et al.*, 1965a), and kanamycin (Nishimura *et al.*, 1962).

It has been reported (Cox *et al.*, 1964; Davies *et al.*, 1965a) that streptomycin and spectinomycin bind to the 30-S subunits of *E. coli* ribosomes and cause a "misreading" of synthetic polyribonucleotides. This effect can be abolished by substituting the 30-S subunits obtained from sensitive cells with those obtained from cells resistant to the antibiotic. The "misreading" induced by these antibiotics is made evident by a stimulation of the incorporation of amino acids which normally are not coded for by the polyribonucleotides employed. Furthermore, a stimulation of the incorporation of the "correct" amino acids has also been reported (Davies *et al.*, 1965b) for many aminoglycosidic antibiotics.

The results reported in the present investigation show that Gentamicin strongly stimulates the incorporation of amino acids directed by natural m-RNA's in *E. coli* extracts. (Incidentally, no stimulation was evident when *E. coli* ribosomal ribonucleic acid (r-RNA) was used as a messenger.) When the cell-free protein synthesis was directed by poly U, the incorporation of isoleucine was strongly stimulated by Gentamicin at all tested s-RNA concentrations. On the other hand, the incorporation of phenylalanine was inhibited by Gentamicin at low but not at high concentrations of s-RNA.

The two main effects of the drug, namely, the stimulation of amino acid incorporation in the presence of endogenous messengers and the stimulation of "wrong" amino acid incorporation with synthetic polyribonucleotides, may be considered different consequences of the same mechanism of interaction between Gentamicin and the *E. coli* translating system. Our experiments, however, have demonstrated that incorporation of amino acids by a cell-free system obtained from a strain resistant to Gentamicin was not stimulated when the synthesis was directed by endogenous m-RNA's. Furthermore, the cellular fraction which is responsible for the resistance to the stimulatory effect of the antibiotic appears to be the 105,000*g* supernatant enzymes.

Such results would suggest that resistance to the antibiotic is due to a mutation affecting a nonribosomal protein. In addition, such results suggest also that, even though the site of attachment of the drug may be on other cellular components (*e.g.*, ribosomes, as the misreading effect seems to indicate), the mechanism of action of Gentamicin involves an interaction of this site with the 105,000*g* supernatant enzymes. In the presence of Gentamicin this interaction results in an increase of amino acid incorporation by cell-free extracts.

Since resistance is achieved by a single-step mutation, a single enzyme should be affected. It is unlikely then that the activating enzymes are involved in the resistance mechanism, as our results seem to indicate (Figure 9). Gentamicin may interfere in the interaction between the amino acid polymerizing enzymes and the aminoacyl-s-RNA–m-RNA–ribosome complex, although we have not provided experimental evidence supporting this hypothesis.

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