Davies, J., Gorini, L., & Davis, B. D. (1965) *Mol. Pharmacol. 1*, 93.
Davies, J., Benveniste, R., Kvitek, K., Ozanne, B., & Yamada, T. (1969) *J. Infect. Dis. 119*, 351.
Davis, B. D., Tai, P.-C. & Wallace, B. J. (1974) in *Ribosomes* (Nomura, M., Tissieres, A., & Lengyel, P., Eds.) p 771, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Dubnoff, J. S., & Maitra, U. (1971) *Methods Enzymol. 20*, 248.
Funatsu, G., & Wittmann, H. G. (1972) *J. Mol. Biol. 68*, 547.
Funatsu, G., Nierhaus, K., & Wittmann-Liebold, B. (1972) *J. Mol. Biol. 64*, 201.
Gesteland, R. F. (1966) *J. Mol. Biol. 16*, 67.
Gorini, L. (1974) in *Ribosomes* (Nomura, M., Tissières, A., & Lengyel, P., Eds.) Cold Spring Harbor Monograph Series, p 791, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4135.
Kaltschmidt, E., & Wittmann, H. G. (1970) *Anal. Biochem. 36*, 401.
Kurland, C. G., Rigler, R., Ehrenberg, M., & Blomberg, C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4248.
Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80*, 575.
Lando, D., Cousin, M. A., Ojasoo, T., & Raynaud, J. P. (1976) *Eur. J. Biochem. 66*, 597.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Milanesi, G., & Ciferri, O. (1966) *Biochemistry 5*, 3926.
Modolell, J., & Davis, B. D. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1279.
Ninio, J. (1974) *J. Mol. Biol. 84*, 297.
Pestka, S. (1977) in *Molecular Mechanisms of Protein Biosynthesis* (Weissbach, H., & Pestka, S., Eds.) pp 467–563, Academic Press, London and New York.
Pestka, S., Marshall, R., & Nirenberg, M. W. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 639.
Piepersberg, W., Böck, A., & Wittmann, H. G. (1975) *Mol. Gen. Genet. 140*, 91.
Pongs, O., Nierhaus, K. H., Erdmann, V. A., & Wittmann, H. G. (1974) *FEBS Lett., Suppl. 40*, 28.
Rosset, R., & Gorini, L. (1969) *J. Mol. Biol. 39*, 95.
Schreiner, G., & Nierhaus, K. (1973) *J. Mol. Biol. 81*, 71.
Strigini, P., & Gorini, L. (1970) *J. Mol. Biol. 47*, 517.
Tai, P. C., & Davis, B. D. (1979) *Biochemistry 18* (accompanying paper).
Thorbjarnardôttir, S. H., Magnûsdôttir, R. A., Eggertsson, G., Kagan, S. A., & Andrêsson, O. S. (1978) *Mol. Gen. Genet. 161*, 89.
Weber, K., & Konigsberg, W. (1975) in *RNA Phages* (Zinder, N. D., Ed.) Cold Spring Harbor Monograph Series, p 51, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Wittmann, H. G., & Wittmann-Liebold, B. (1974) in *Ribosomes* (Nomura, M., Tissières, A., & Lengyel, P., Eds.) Cold Spring Harbor Monograph Series, p 115, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Wittmann, H. G., Stöffler, G., Piepersberg, W., Buckel, P., Ruffler, D., & Böck, A. (1974) *Mol. Gen. Genet. 134*, 225.

Triphasic Concentration Effects of Gentamicin on Activity and Misreading in Protein Synthesis†

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ABSTRACT: Gentamicin is shown to exert a triphasic concentration effect on peptide synthesis in vitro with natural messengers. Low concentrations (up to 2 μM) caused slowing and a decrease in total synthesis, but little misreading (assayed with extracts lacking Glu-tRNA); the inhibition was greater with an initiating system (with phage RNA as messenger) than with pure chain elongation on purified endogenous polysomes of *Escherichia coli*. Moderate concentrations (up to 100 μM) slowed synthesis less, markedly increased its duration in the noninitiating system, and strongly stimulated misreading; at optimal concentrations total synthesis was even greater than normal. Moreover, with phage RNA these concentrations increased the synthesis of large polypeptides. We conclude that binding of gentamicin to its first site causes inhibition but little misreading; binding to additional site(s) partly reverses the inhibition by first-site binding and markedly stimulates misreading, and the misreading appears to favor "readthrough" of termination codons. In the third phase (>100 μM) synthesis is slowed again but the pattern of misreading does not appear to be altered; this effect need not involve a specific further action on the ribosome.

Among the aminoglycoside antibiotics that inhibit protein synthesis and can also cause extensive misreading (Davies et al., 1965; reviewed by Pestka, 1977), streptomycin (Str)[1] has been studied most extensively. Its blockade of initiating ribosomes and its slowing and misreading of ribosomes already engaged in chain elongation are identical in their concentration range (reviewed by Davis et al., 1974; Wallace et al., 1978). These actions are consistent with the evidence that ribosomes

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[1] Abbreviations used: IF, initiation factors; Gm, gentamicin C mixtures containing C_1, C_{1a}, and C_2; Str, streptomycin; $NaDodSO_4$-gel electrophoresis, polyacrylamide gel electrophoresis containing 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer; ts, temperature sensitive.

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bind a single molecule (Chang & Flaks, 1972).

It has generally been assumed that all aminoglycosides have similar mechanisms of action. However, with gentamicin (Gm), which causes the highest observed level of misreading of poly(U) (Davies et al., 1965), variations in concentration over a broad range markedly affect the degree of misreading (Davies & Davis, 1968). This finding suggested that Gm may act on more than one site on a ribosome. Gm also differed from Str in inhibiting initiating systems incompletely: it decreased translation of phage R17 RNA only 50–60% (Davies et al. 1969), while Milanesi & Ciferri (1966) have reported that it actually stimulated translation of tobacco mosaic virus RNA.

Since the actions of several other classes of antibiotics have been illuminated by comparison of their effects on initiating ribosomes and on purified polysomes that carry out only chain elongation (reviewed by Davis et al., 1974), we have studied the effect of Gm on these two systems. With both it exerts a triphasic concentration effect: at low concentrations chain elongation is markedly inhibited; at higher concentrations the inhibition diminishes, while misreading is enhanced and synthesis is prolonged; and at still higher concentrations inhibition increases again.

Materials and Methods

Bacteria and Fractions. RNase I⁻ *Escherichia coli* strain MRE600 was grown at 37 °C, and purified endogenous polysomes free of initiation factors (IF) were prepared by dry-ice grinding and Sepharose 4B chromatography as described (Tai et al., 1973). Washed ribosomes (washed with 1 M NH_4Cl), crude IF, and supernatant enzyme fraction (S100) from MRE600 were prepared as described (Tai et al., 1973) and stored in small quantities at −76 °C. Temperature-sensitive (ts) *E. coli* mutant 5F2 (ArgA⁻, GluS-ts) from Kaplan et al. (1973), grown at 30 °C with aeration in L broth supplemented with the salts of medium A (Davis & Mingioli, 1950), was incubated at 42 °C for 30 min to inactivate the ts Glu-tRNA synthetase and to exhaust Glu-tRNA. The cultures were then poured over ice, and the S100 and IF fractions were prepared from the cells and stored at −76 °C.

Reagents and Buffers. Buffers and phage R17 RNA were prepared as described (Tai et al., 1973). R17 coat protein was isolated as described by Hofstetter et al. (1974); it migrated as a single band in $NaDodSO_4$-gel electrophoresis (see below). Rabbit antiserum against the coat protein was induced by injecting 1 mg of purified coat protein mixed with complete Freund's adjuvant according to established procedures (cf. Furano, 1975). An equimolar mixture of 20 ^{14}C-labeled amino acids, each at 10 Ci/mol, was prepared by adjusting each amino acid to the specific activity desired. All of the radioactive amino acids were from New England Nuclear. Gentamicin C sulfate mixture (average M_r of 460) and subcomponents C_1, C_{1a}, and C_2 were from Dr. M. J. Weinstein of Schering Corp.; streptomycin sulfate was from E. R. Squibb Co. Other reagents (reagent grade) were obtained from commercial sources.

In Vitro Protein Synthesis. Polypeptide synthesis was carried out at 36 °C as described (Tai et al., 1973). The reaction mixtures, usually in 0.1 mL, contained 50 mM Tris-HCl (pH 7.6), 60 mM NH_4Cl, 20 mM KCl, 2 mM dithiothreitol, 1 mM ATP–Tris, 0.02 mM GTP, 5 mM potassium phosphoenolpyruvate, 3 μg of pyruvate kinase, 10 μL of S100 fraction (optimal amount, ca. 80 μg of protein), 9 mM $Mg(OAc)_2$, and a mixture of 20 ^{14}C-labeled amino acids (unless otherwise specified, each at 25 mM and 10 Ci/mol). For study of chain elongation 50 μg of purified polysomes was added (assuming 16.6 A_{260} units = 1 mg). For study of initiating systems 50 μg of washed ribosomes (activated at 50 °C for 2 min before use) was added together with phage R17 RNA (500 μg/mL) and crude IF (200 μg/mL) (the amounts yielding maximal activity). Samples were taken at intervals as specified, incorporation was stopped by the addition of 2mL of 5% trichloroacetic acid containing 200 μg/mL of Casamino acid mixture, and the precipitate was collected and assayed for radioactivity in toluene-based scintillation fluid with a Searle Isocap scintillation counter. Background (200–600 cpm) without incubation was subtracted from all data presented.

Formation of Initiation Complexes. The formation of initiation complexes was assayed on Millipore filters (HAWP; 0.45 μm) with f[^{3}H]Met-tRNA; the assay procedures and preparation of f[^{3}H]Met-tRNA were as described previously (Wallace et al., 1974) except that 1 mg/mL of R17 RNA was used.

Disc Gel Electrophoresis. To separate the labeled peptides from unreacted radioactive aa-tRNA and from the attached tRNA, the samples were precipitated with 5% trichloroacetic acid, dissolved in 1 mL of 0.3 N NaOH, incubated at 36 °C for 10 min, and mixed with 1.5 mL of 10% trichloroacetic acid. The precipitates were dissolved and subjected to $NaDodSO_4$/phosphate/polyacrylamide gel electrophoresis, as described by Shapiro et al. (1967) and modified by Weber & Osborn (1969), in 7.5% gels at 8 mA per tube for 5–7 h. Slices (1 mm) were added to 8 mL of toluene-based scintillation fluid containing 3% of a 5% H_2O/95% Protosol (New England Nuclear) mixture, the capped vials were incubated at 37 °C overnight or at 60 °C for 3 h, and the radioactivity was determined.

Results

Effects of Gm Concentration on Translation by Initiating and by Elongating Ribosomes. We have previously shown that purified polysomes carry out peptide chain elongation (and termination) on endogenous mRNA but do not reinitiate (Tai et al., 1973). Varying concentrations of Gm affected total incorporation by this system, measured at 15 min, in a surprisingly complicated, multiphasic way. Up to 0.4 μM (with 0.2 μM ribosomes) Gm was inhibitory, but only up to about 40% (Figure 1). From 0.4 to 2 μM the inhibition remained more or less constant. Above 2 μM the inhibition gradually diminished, and in fact at 20–60 μM the total incorporation was even slightly higher than in the absence of the antibiotic. Above 60 μM the total incorporation again decreased. This triphasic effect was virtually identical for incorporation measured either with [^{14}C]valine or with a mixture of 20 ^{14}C-labeled amino acids. In the latter experiments all 20 amino acids had the same specific activity, and so the incorporation observed represents the actual total peptide elongation, including amino acid substitutions.

Translation initiating on R17 RNA (measured at 30 min) also showed a triphasic effect with increasing Gm concentration, except that the inhibition was greater, reaching 65% (with 20 ^{14}C-labeled amino acids) at 2 μM (Figure 1). The other two phases paralleled those observed for chain elongation but with less stimulation in the second phase. With [^{14}C]-valine, which is especially prevalent in the phage coat protein (the normal main product of in vitro translation), the observed inhibition is seen to be greater, especially at higher Gm concentrations. The difference between the two indexes of incorporation evidently reflects a differential effect of Gm-induced misreading either on specific amino acid replacements or on the portions of the phage RNA being translated.

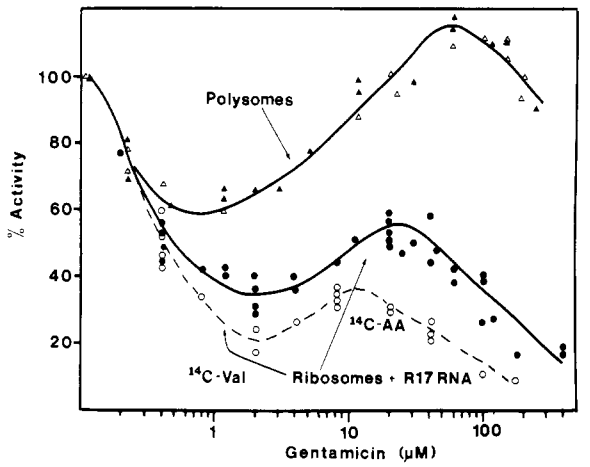

FIGURE 1: Effect of Gm concentration on protein synthesis. For chain elongation on endogenous polysomes the reaction mixtures contained purified IF-free polysomes and either 20 ^{14}C-labeled amino acids each at 10 Ci/mol (▲) or 30 μM [^{14}C]valine (80 Ci/mol) and 50 μM of 19 other unlabeled amino acids (△); Gm concentration was as indicated. After incubation for 15 min incorporation was measured as described under Materials and Methods. For the translation of R17 RNA the mixtures contained NH_4Cl-washed ribosomes, IF, R17 RNA, and either 20 ^{14}C-labeled amino acids (●) or [^{14}C]valine plus unlabeled amino acids (○) as above, with Gm as indicated. The incubation was for 30 min; 100% activity ranged from 20000 to 60000 cpm.

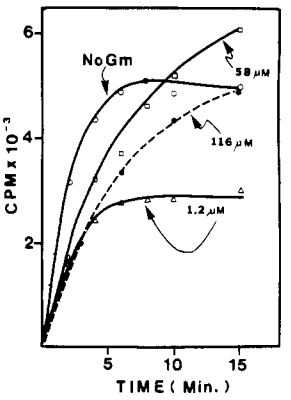

FIGURE 2: Time course of incorporation on endogenous polysomes in the presence of Gm. The incorporation, with 20 ^{14}C-labeled amino acids and with Gm as specified, was studied as described in Figure 1; 30-μL samples were analyzed.

Gm thus resembles Str in having a more inhibitory effect on initiating than on chain-elongating ribosomes (Luzzatto et al., 1969; Wallace et al., 1973), but it differs in its triphasic concentration effect and in the incomplete inhibition of initiating ribosomes.

This triphasic effect seemed unlikely to be due to different actions of the three components (Cooper & Mariglian, 1969) of Gm, since Davies et al. (1969) have shown that translation of R17 RNA was inhibited to a similar degree by the individual components. We have found (data not shown) that with either endogenous polysomes or R17 RNA pure C_1, C_{1a}, and C_2 all exhibited similar triphasic concentration effects on translation.

Kinetics of Chain Elongation in the Presence of Various Gm Concentrations. Kinetic studies showed interesting differences in the effects of Gm at different concentrations, representative of the phases noted above (Figure 2). Without antibiotic the nonreinitiating system, completing its nascent chains, incorporated linearly for several minutes and ceased by 6 min, as noted previously (Tai et al., 1973). Gm at 1.2 μM slowed incorporation ca. 50%, and incorporation again

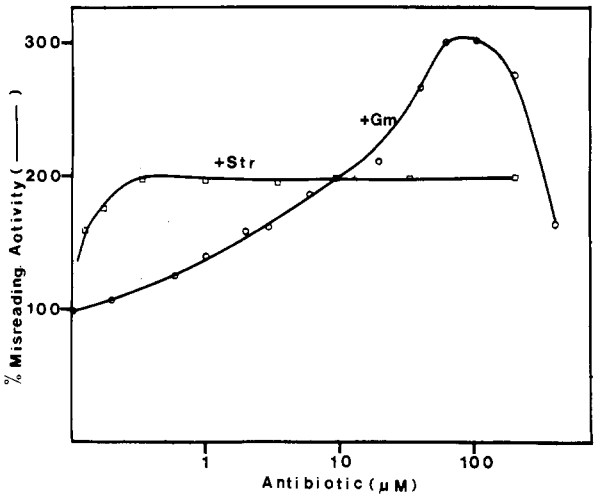

FIGURE 3: Stimulation of misreading by Gm. The reaction mixtures were as in Figure 2, except that 5F2 S100 (with ts Glu-tRNA synthetase) was used, with Gm (○) or Str (□) at the concentration indicated. The incubation was for 15 min, and 50-μL samples were analyzed. The activity without antibiotic (100%, reflecting intrinsic misreading) was 2879 cpm.

ceased at about 6 min; hence, the total was correspondingly diminished. However, at 58 μM Gm (the second phase) incorporation was slowed less, and it was still continuing at the termination of the experiment, 15 min later. (In separate experiments incorporation had ceased by 20 min; hence, the net stimulation of translation by endogenous polysomes observed in Figure 1 was slightly underestimated.) Gm at 116 μM (the third phase) had an effect much like that at 58 μM, except that incorporation was somewhat slower.

The slightly faster chain elongation observed in the second phase (Figure 2) suggests that even before the first binding site is saturated binding at an additional site(s) begins and partly reverses the inhibitory effect of first-site binding.

Misreading of Endogenous Polysomes Induced by Gm. The increased incorporation in the second phase could be due to several mechanisms: reinitiation induced by the antibiotic in the absence of IF, stabilization of messenger, or extensive misreading with preferential suppression of termination. To test the effect of Gm concentration on misreading of natural messenger we used, a mutant (5F2) with a ts Glu-tRNA synthetase (Tai et al., 1978). With S100 from such a strain, at 36 °C, the incorporation of the other 19 labeled amino acids depends largely on the misreading of other aa-tRNAs as Glu-tRNA.

Str, as shown previously (Tai et al., 1978), stimulated this misreading-dependent incorporation, reaching a plateau at the low concentration of 0.3 μM (Figure 3). In this concentration range Gm, in contrast, had little effect on misreading, though it inhibited incorporation with a complete system. However, in the second concentration phase Gm markedly stimulated misreading: at 10 μM the incorporation without Glu-tRNA reached the level of the plateau observed with Str, and at 70–100 μM it was further doubled. This extensive stimulation of misreading had the same range of concentration dependence as the increased incorporation in the presence of all available aa-tRNAs (Figure 1, polysomes), suggesting a common mechanism for the two effects.

In the presence of 100 μM Gm (i.e., a high concentration) the kinetics of total incorporation without Glu-tRNA (data not shown) were almost identical with those observed with Glu-tRNA (Figure 2). It thus appears that with sufficient Gm, just as with Str over a broader concentration range (Tai

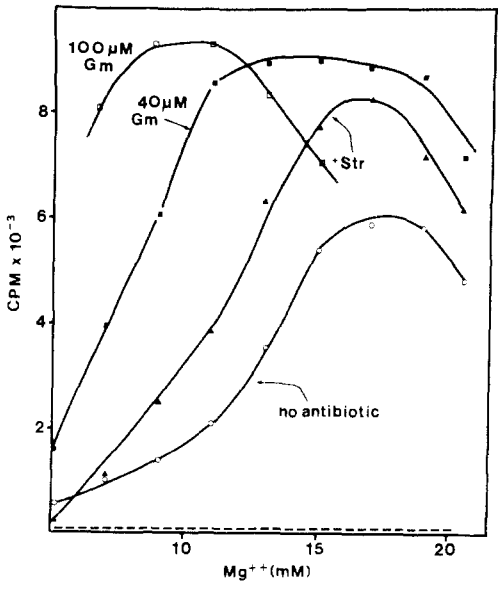

FIGURE 4: Effects of Mg^{2+} concentration on the misreading of endogenous polysomes. The reaction mixtures (0.05 mL) were as in Figure 3, with Mg^{2+} as indicated and either 100 μM Gm (□), 40 μM Gm (■), 34 μM Str (▲), or no antibiotic (○); incubation was for 15 min. The inactivity of Glu-tRNA synthetase is indicated by the lack of incorporation of [^{14}C]glutamate with 19 unlabeled amino acids, with or without antibiotic (dashed line).

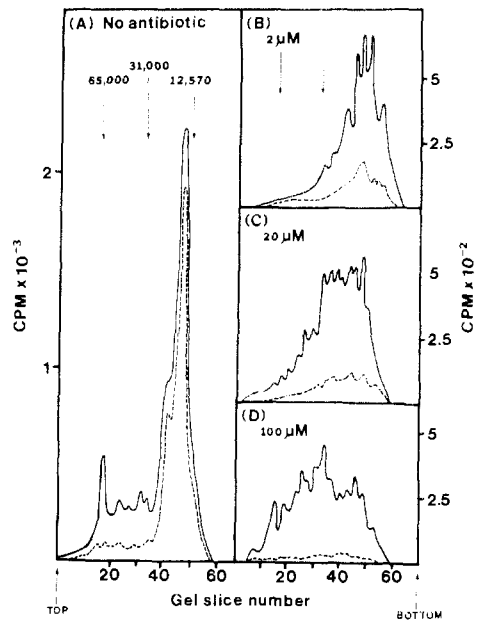

FIGURE 5: Product of translation of R17 RNA in the presence of Gm. The reaction mixtures were as in Figure 1 for the initiating system with MRE600 S100, with Gm at (A) 0 μM, (B) 2 μM, (C) 20 μM, or (D) 100 μM. Incubation was for 30 min. To determine total translation products (solid line) 50 μL of each reaction mixture was precipitated with 5% trichloroacetic acid, and the precipitates were further treated as described under Materials and Methods for gel electrophoresis. To determine coat protein by immunoreaction (dashed line) 2 μg of unlabeled R17 coat protein was added to 50 μL of each reaction mixture, the volume was adjusted to 0.2 mL with buffer (0.01 M NaH_2PO_4, pH 7.4, 0.14 M NaCl), and 10 μL of anticoat protein serum (predetermined optimal amount) was added. The mixtures were incubated at 36 °C for 1 h and placed at 0 °C overnight, and the precipitates were collected by centrifugation, washed once with the same buffer, and prepared for and subjected to gel electrophoresis. To simplify figures, data on counted slices are presented as tracing lines.

et al., 1978), the substitution of noncognate aa-tRNA is almost as extensive as the correct incorporation of Glu-tRNA.

At very high concentrations further inhibition was seen without Glu-tRNA, and it closely paralleled that with Glu-tRNA. The increased inhibition of synthesis in this third phase thus does not involve any observable change in the extent of misreading.

Dependence of Antibiotic-Induced Misreading on Mg^{2+} Concentration. As we have shown previously (Tai et al., 1978), the intrinsic misreading by endogenous polysomes in the absence of Glu-tRNA, like that observed earlier with poly(U) (Davies et al., 1964), increases markedly with Mg^{2+} up to 16–18 mM, and the enhancing effect of Str is constant over a wide range of concentrations. As Figure 4 shows, Str superposes a rather constant additional misreading on that induced by various concentrations of Mg^{2+}, whereas high concentrations of Gm exert their maximal misreading effect at much lower concentrations of Mg^{2+}: 100 μM Gm reached its peak effect at 8–10 mM Mg^{2+}, compared with 16 mM for Str.

Size Distribution of Products of Translation. Since Gm (unlike Str) allows considerable initiation of translation on phage RNA, this system could be used to study the effect of Gm on the formation of defined products. The major protein synthesized without antibiotic (Capecchi & Webster, 1975) was coat protein, identified by its M_r of 14 000 in $NaDodSO_4$–gel electrophoresis and further by precipitation with anticoat serum (Figure 5A, dashed line). With 2 μM Gm, which reduced synthesis severely, several peaks somewhat larger than coat protein appeared, and a reduced but still substantial amount of material reacted with anticoat serum (Figure 5B). At 20 μM, in contrast, Gm induced the formation of much larger peptides, some of which partially reacted with anticoat serum (Figure 5C); at 100 μM the average size of the peptides increased further, and they did not react with anticoat serum (Figure 5D).

In similar experiments with the system lacking Glu-tRNA there was small but substantial peptide synthesis without antibiotic as previously observed (Tai et al., 1978); it reflects intrinsic misreading. The product included a small amount of modified coat protein (with normal M_r and immunological reactivity despite the replacement of glutamate). With 100 μM Gm the peptides formed without Glu-tRNA were very similar in size distribution to those formed with Glu-tRNA (data not shown), just as they were similar in amount (as noted above).

The products of translation of endogenous polysomes were also examined. In the absence of Glu-tRNA the limited synthesis obtained without antibiotic yielded mostly small peptides, and 100 μM Gm greatly enhanced the amount and size of the peptides synthesized, yielding a pattern almost identical with that observed with Glu-tRNA available (data not shown).

The increased sizes observed in the presence of Gm, with both messengers, indicate that the enhancement of incorporation by Gm in the absence of Glu-tRNA is largely due to misreading (i.e., replacement of the missing Glu-tRNA by other aa-tRNA's in chain elongation) rather than to stimulation of reinitiation (i.e., the formation of additional short peptide chains).

Formation of Initiation Complexes. The formation of initiation complexes, as measured by f[^{3}H]Met-tRNA binding, was inhibited 50% by 2 μM Gm and completely by 10 μM; no multiphasic concentration curve was observed (data not shown). This complete inhibition at a high concentration appears to contradict the substantial incorporation observed with an initiating system at the same concentration (Figure 1). However, the initiation complexes formed in the presence

of Gm have been reported to be unstable (Okuyama et al., 1972), and so they might have been formed but not recovered under our conditions.

Discussion

Amino acid incorporation by purified endogenous polysomes, which finish their nascent chains but do not reinitiate, exhibits a multiphasic concentration effect of Gm. At low concentrations (up to 4 μM) Gm decreases the total amount (Figure 1) and the rate (Figure 2) of synthesis but causes little misreading. At higher concentrations the inhibition is partly reversed, and misreading, measured by synthesis in the absence of Glu-tRNA, is markedly increased. At still higher concentrations (>60 μM) a third phase is observed, with greater slowing but with no conspicuous change in prolongation of synthesis or in misreading.

Since the actions of Gm vary over a much wider concentration range than that of a first-order reaction, and since the first and the second phases differ markedly in prolongation of synthesis and in misreading, it must be inferred that at increasing concentrations Gm, unlike Str, binds to additional site(s). The first binding has the primary action, like Str (Wallace et al., 1973), of slowing chain elongation, but it causes much less misreading than Str (Figure 3). Binding to the subsequent site(s) partly reverses the slowing effect of the first binding and induces a stronger misreading activity, with the maximal misreading effect at a much lower Mg^{2+} concentration than that observed with Str (Figure 4). The third phase, in contrast, shows only increased slowing, with no evident change in the pattern of synthesis; hence, this effect does not necessarily imply binding to ribosomal sites of still lower affinity but might be due to complexing of this polycationic antibiotic, at high concentrations, with other components of the system. Multiple-site binding of Gm was suggested earlier by Davies & Davis (1968) on the basis of concentration effects on the misreading of synthetic message, and S. Perzynski & J. Davies (personal communication) have recently demonstrated cooperative binding of 3–5 molecules of radioactive Gm to each ribosome.

It seems clear that prolongation of synthesis by higher concentrations of Gm is largely or completely due to a misreading that results in readthrough of termination more often than in premature termination. This interpretation is supported by several observations: the marked stimulation of synthesis in the absence of Glu-tRNA (Figure 3); the closely parallel concentration dependence of this stimulation of misreading in the incomplete system and the stimulation of synthesis with a complete system; and the demonstration (see also Kühberger et al., 1979) that the latter stimulation involves a marked increase in the size of the peptides produced (Figure 5). However, while the shift in size is consistent with readthrough, it also could be due to the reported preferential effect of Gm on initiation of synthesis of phage maturation protein, compared with the smaller coat protein (Okuyama & Tanaka, 1972).

The inhibition of initiation-dependent R17 RNA translation by Gm, in contrast to Str, is only partial. It seems possible that binding to the first site completely inhibits initiation but that second-site binding, which decreases the inhibition and increases misreading, begins before the first site is saturated. This view is supported by the existence of mutant ribosomes (Kühberger et al., 1979); our unpublished observations) on which Gm completely inhibits protein synthesis initiating on R17 RNA and shows much reduced misreading of poly(U).

Gm has been observed to have a biphasic concentration effect also on translocation (Cabanas et al., 1978). This antibiotic evidently has pleiotropic effects on recognition and on translocation that vary with binding at successive sites. Another ligand, EFG, has also been reported to influence the fidelity of translation (Gavrilova et al., 1976) as well as translocation. The recent demonstration of two stages in recognition on the A site (Thompson & Stone, 1977) suggests that antibiotics might influence the accuracy of either the first, reversible stage, or the second, GTP-linked proofreading stage.

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References

Cabanas, M. J., Vazquez, D., & Modolell, J. (1978) *Biochem. Biophys. Res. Commun. 83*, 991.

Capecchi, M. R., & Webster, R. E. (1975) in *RNA Phages* (Zinder, N. D., Ed.) p 279, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Chang, F. N., & Flaks, J. G. (1972) *Antimicrob. Agents Chemother. 2*, 294.

Cooper, D. J., & Marigliano, H. M. (1969) *J. Infect. Dis. 119*, 342.

Davies, J., & Davis, B. D. (1968) *J. Biol. Chem. 243*, 3312.

Davies, J., Gilbert, W., & Gorini, L. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 883.

Davies, J., Gorini, L., & Davis, B. D. (1965) *Mol. Pharmacol. 1*, 93.

Davies, J., Benveniste, R., Kvitek, K., Ozanne, B., & Yamada, T. (1969) *J. Infect. Dis. 119*, 351.

Davis, B. D., & Minigioli, E. S. (1950) *J. Bacteriol. 60*, 17.

Davis, B. D., Tai, P.-C., & Wallace, B. J. (1974) in *Ribosomes* (Nomura, M., Tissieres, A., & Lengyel, P., Eds.) p 771, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Furano, A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4780.

Gavrilova, L. P., Kostiashkina, O. E., Koteliansky, V. E., Ritkevitch, N. M., & Spirin, A. S. (1976) *J. Mol. Biol. 101*, 537.

Hofstetter, H., Monstein, H.-J., & Weissmann, C. (1974) *Biochim. Biophys. Acta 374*, 238.

Kaplan, S., Atherly, A. G., & Varrett, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 689.

Kühberger, R., Piepersberg, W., Petzet, A., Buckel, P., & Bock, A. (1979) *Biochemistry 18* (accompanying paper).

Luzzatto, L., Apirion, D., & Schlessinger, D. (1969) *J. Bacteriol. 99*, 206.

Milanesi, G., & Ciferri, O. (1966) *Biochemistry 5*, 3926.

Okuyama, A, & Tanaka, N. (1972) *Biochem. Biophys. Res. Commun. 49*, 951.

Okuyama, A., Watanabe, T., & Tanaka, N. (1972) *J. Antibiot. 25*, 212.

Pestka, S. (1977) in *Molecular Mechanisms of Protein Biosynthesis* (Weissbach, H., & Pestka, S., Eds.) p 467, Academic Press, London and New York.

Shapiro, A. L., Vinuela, E., & Maizel, T. V. (1967) *Biochem. Biophys. Res. Commun. 28*, 815.

Tai, P.-C., Wallace, B. J., Herzog, E. L., & Davis, B. D. (1973) *Biochemistry 12*, 609.

Tai, P.-C., Wallace, B. J., & Davis, B. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 275.

Thompson, R. C., & Stone, P. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 198.

Wallace, B. J., Tai, P.-C., Herzog, E. L., & Davis, B. D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1234.
Wallace, B. J., Tai, P.-C., & Davis, B. D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1934.
Wallace, B. J., Tai, P.-C., & Davis, B. D. (1978) in *Molecular Mechanisms of Antibiotic Action* (Hahn, F. E., Ed.) Academic Press, New York, in press.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Extracellular Labeling of Growing Secreted Polypeptide Chains in *Bacillus subtilis* with Diazoiodosulfanilic Acid†

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ABSTRACT: Studies of the mechanism of protein secretion in a Gram-positive bacterium, *Bacillus subtilis*, yielded results very similar to those previously obtained with a Gram-negative organism: nascent chains protruding from protoplasts could be labeled extracellularly; the labeled chains could be recovered on polysomes isolated from the membrane–polysome fraction; they could be released by puromycin, low Mg^{2+}, or chain completion; the completed chains include a known secreted protein (α-amylase); and the ribosomes appear to be attached to membrane solely by their nascent chains. The reagent used for extracellular labeling, [^{125}I]diazoiodosulfanilic acid, yielded severalfold more specific labeling of the nascent chains (7–10% of the total cellular labeling and one-fourth to one-third of that of the membrane–polysome fraction) than was obtained earlier with another nonpenetrating reagent.

The presence of ribosomes bound to the endoplasmic reticulum, in animal cells that secrete proteins, suggested that these proteins cross the membrane as growing chains (reviewed by Palade, 1975). This model was supported by the finding that extracts of a myeloma cell synthesized an immunoglobulin chain as a larger precursor, with an additional N-terminal "signal" sequence (Milstein et al., 1972) of predominantly hydrophobic residues (Schechter et al., 1975). The formation of such a precursor, its cleavage by an enzyme in the membrane, and in many cases the sequence of the additional segment have now been demonstrated for many proteins secreted by animal cells (e.g., Blobel & Dobberstein, 1975; Devillers-Thiery et al., 1975) and more recently for proteins secreted by bacteria (Inouye et al., 1977; Inouye & Beckwith, 1977). These findings strongly suggested that such signal segments (Blobel & Sabatini, 1971) play a role in initiating secretion, but they do not establish whether the chain that follows is folded and released from the ribosome before or after transfer across the membrane. With spheroplasts of *Escherichia coli* we were able to demonstrate directly that a periplasmic protein is secreted as a growing chain (Smith et al., 1977): such chains could be extracellularly labeled with a reagent ([^{35}S]acetylmethionyl methyl phosphate sulfone, AMMP[1]) that acylates amino groups. Moreover, in this organism polysomes appear to be attached to membrane solely via nascent chains (Smith et al., 1978a), in contrast to results reported for animal cells (Adelman et al., 1973; Sabatini & Kreibich, 1976) and for chloroplasts (Chua et al., 1976).

The present work extends these studies, with very similar results, to a Gram-positive organism, *Bacillus subtilis*. In addition, since only 2% of the bound label was attached to growing chains in our studies with AMMP (Smith et al., 1977), we have explored the use of a reagent, diazotized [^{125}I]iodosulfanilic acid (DSA), that reacts only with histidine and tyrosine residues (reviewed by Carraway, 1975) and have obtained three- to fourfold increase in the specificity of labeling.

Materials and Methods

Bacteria and Protoplasts. *B. subtilis* cells of ATCC strain 6051a, constitutive in the synthesis of α-amylase, were grown at 37 °C with vigorous aeration (unless otherwise indicated) in minimal medium A (Davis & Mingioli, 1950) supplemented with 0.4% glucose and 0.2% casamino acids. To stabilize polysomes chloramphenicol (200 μg/mL) was added to 25 mL of an exponentially growing culture (5×10^8 cells/mL), which was poured onto excess ice. The cells were pelleted by brief centrifugation in the cold and resuspended in 2 mL of 100 mM Tris-HCl, pH 8.0, with 25% w/v sucrose. Protoplasts were formed by adding 400 μg of lysozyme and incubating at 37 °C for 10 min. Conversion was usually greater than 95% as monitored by microscopy.

Extracellular Labeling of Protoplasts. The protoplasts were centrifuged and resuspended in 0.5 mL of 10 mM NaH_2PO_4, pH 7.5, with 25% sucrose. Unless otherwise indicated, 50 μCi of [^{125}I]DSA (specific activity ~2000 Ci/mmol) was dried under vacuum, converted to the diazonium salt by incubation for 10 min at 0 °C with 10 μL of 0.05 mM $NaNO_2$ and 10 μL of 1 mM HCl, and added to the protoplast suspension derived from 1.2×10^{10} cells (final DSA concentration, 6 μM). After incubation for 15 min at 0 °C with gentle shaking, the

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[1] Abbreviations used: AMMP, acetylmethionyl methyl phosphate sulfone; DSA, diazoiodosulfanilic acid; $NaDodSO_4$ gel electrophoresis, gel electrophoresis containing 10% polyacrylamide, 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer; EF-G, bacterial elongation factor G; RRF, ribosome release factor; buffer A, 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM $Mg(OAc)_2$.

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