

## INHIBITION OF THE INITIAL DIPEPTIDE SYNTHESIS OF GLOBIN CHAINS BY THE ANTIBIOTIC ENOMYCIN IN THE RETICULOCYTE LYSATE

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During inhibition of protein synthesis by the antibiotic enomycin at less than 5  $\mu\text{M}$  in the reticulocyte lysate system, polyribosomes disaggregated and the 80S ribosomes accumulated. At these concentrations little inhibition of chain elongation and release from the ribosomes was demonstrated. Enomycin caused an increase in the amount of 80S initiation complex as well as the 40S ribosomal subunit-Met-tRNA<sub>f</sub> complex. The former complex could react with puromycin under the inhibiting conditions. Val-tRNA binding to the 80S ribosomes was not decreased by the antibiotic. However pactamycin-induced accumulation of the initial dipeptide (fMet-Val) was inhibited when the system was preincubated with enomycin and fMet-tRNA<sub>f</sub>. Thus the preferential inhibition of the initial phase of protein synthesis by enomycin is made evident by its inhibition of the initial dipeptide synthesis.

Enomycin is a basic polypeptide antibiotic (molecular weight 11,000) produced by a streptomycetes<sup>1)</sup>. The antibiotic exhibits a tumor-inhibitory activity against EHRlich carcinoma and sarcoma 180 in mice but no antimicrobial activity<sup>1)</sup>. Its primary mode of action is inhibition of protein synthesis in animal cells, but the antibiotic shows little inhibition of bacterial protein synthesis<sup>2,3)</sup>. In those previous studies, however, a cell-free system from EHRlich ascites cells and rat liver was used which mostly supported peptide chain elongation with little chain initiation. Therefore we have examined the inhibition of protein synthesis in the reticulocyte lysate system in which there is active reinitiation of peptide chains. The results indicate that enomycin at low concentrations preferentially inhibits the initial phase of protein synthesis by affecting the initial dipeptide formation.

### Materials and Methods

#### Protein-synthesizing reticulocyte lysate

Preparation of the reticulocytes and the cell-free lysate system were as previously described<sup>4)</sup>. Hemin was added to yield a final concentration of 40  $\mu\text{M}$ . For radioactivity determination aliquots of the reaction mixtures were treated as described<sup>5)</sup>. Trichloroacetic acid-insoluble materials were collected on Whatman glass fiber paper (GF/C) and counted in a liquid scintillation spectrometer.

#### Sucrose density gradient analysis

For sucrose gradient analysis diluted reaction mixtures were layered on 12 ml of a linear 15~30 % (w/w) sucrose gradient in buffer A (10 mM Tris-HCl, pH 7.5, 10 mM KCl and 1.5 mM MgCl<sub>2</sub>) and centrifuged in a Beckman SW41 rotor at 40,000 rpm (280,000  $\times g$ , maximal) for indicated times. After the run the gradient was analyzed as described<sup>6)</sup>. To quantitate the radioactivity associated with the 40S ribosomal subunits and 80S ribosomes radioactivity in the individual fractions (five in number) was totaled.

### Cellulose thin-layer chromatography

The systems were incubated and diluted with 2 volumes of buffer A. The mixture was applied to 5 ml of 1M sucrose in buffer A and centrifuged at 50,000 rpm ( $235,000 \times g$ , max.) for 3 hours. The ribosome pellet was dissolved in 0.2 ml of 2M  $\text{NH}_4\text{OH}$  and the solution was dried *in vacuo*. The dried sample was dissolved again in 20  $\mu\text{l}$  of the alkaline solution with fMet and fMet-Val as internal standards. An aliquot (5  $\mu\text{l}$ ) was applied to a cellulose thin-layer plate (Merck) and the plate was developed with pyridine-acetic acid-butanol-water (18:7:30:6, v/v). The plate was stained with platinum iodide<sup>6)</sup> for location of the markers. The plate was scanned in a chromatogram scanner (Aloka, Model TLC-2B) for radioactivity.

### Preparation of f[<sup>35</sup>S]Met-tRNA<sub>f</sub> and [<sup>3</sup>H]Val-tRNA

f[<sup>35</sup>S]Met-tRNA<sub>f</sub> was prepared by incubating reticulocyte tRNA (unfractionated and deacylated) with *Escherichia coli* aminoacyl-tRNA synthetases and transformylase in the presence of [<sup>35</sup>S]methionine (33 Ci/mole) and Ca-leuovorin as described<sup>7)</sup>. [<sup>3</sup>H]Val-tRNA was also prepared by incubating the tRNA with the reticulocyte aminoacyl-tRNA synthetases and [<sup>3</sup>H]valine (4.8 Ci/mole).

### Materials

Labeled amino acids were obtained from the Radiochemical Center, Amersham, England. Formyl-methionine was from Sigma, U. S. Formyl-methionyl-valine was prepared from methionyl-valine as described<sup>8)</sup>. Enomycin was isolated from a culture filtrate of *Streptomyces mauvecolor*.<sup>1)</sup> Pactamycin was kindly supplied by Upjohn Chemicals, U. S.

## Results

### Inhibition of Protein Synthesis by Enomycin

Protein synthesis as estimated by leucine incorporation was inhibited by enomycin (3 nM) after a lag of one minute (Fig. 1A). Formyl-methionine incorporation into the amino terminus of globin chains from added fMet-tRNA<sub>f</sub><sup>9,10)</sup> was inhibited without a lag period (Fig. 1B). Under these conditions polyribosomes disagggregated and the 80S ribosomes accumulated (Fig. 2),

Fig. 1. Inhibition of protein synthesis by enomycin

The lysate systems (220  $\mu\text{l}$ ) were incubated with [<sup>14</sup>C]leucine (0.03 mM, 62 Ci/mole) (A), and f[<sup>35</sup>S]Met-tRNA<sub>f</sub> ( $7 \times 10^5$  cpm, 33 Ci/mole) (B), in the presence or absence of enomycin (3 nM) at 34°C. A 15  $\mu\text{l}$  aliquot was taken for the assay.

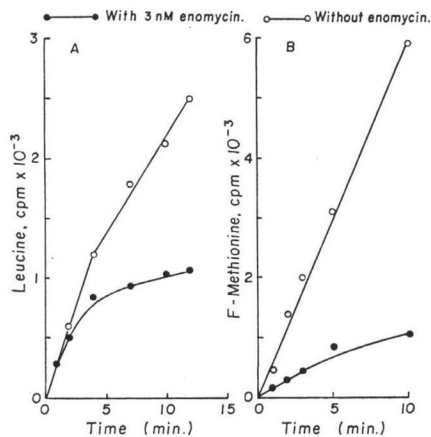


Fig. 2. Effect of enomycin on the ribosome profile in the lysates

The systems (220  $\mu\text{l}$ ) were incubated at 34°C for 7 minutes with or without enomycin (5 nM). The reaction was terminated by chilling and the addition of 200  $\mu\text{l}$  of cold buffer A. A 400  $\mu\text{l}$  aliquot was layered on a sucrose gradient. Centrifugation was for 2 hours at 4°C. (A) Control (unincubated); (B) Control (incubated); (C) Enomycin

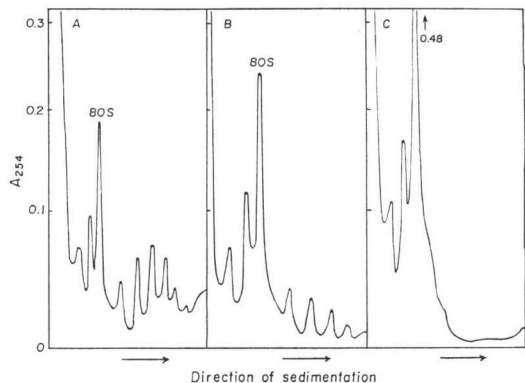


Table 1. Effect of enomycin on chain elongation and release from ribosomes

The lysate which contained [ $^{14}\text{C}$ ]leucine labeled nascent peptides on the polyribosomes was prepared as described<sup>9</sup>. The systems (55  $\mu\text{l}$ ) containing the labeled polyribosomes were incubated at 30°C for 5 minutes with or without enomycin or sparsomycin. After the incubation the mixtures were diluted with 200  $\mu\text{l}$  of buffer A and centrifuged at 50,000 rev/min (235,000  $\times g$ , max) for 2.5 hours to sediment the ribosomes. An aliquot (50  $\mu\text{l}$ ) of supernatant was treated with 5% trichloroacetic acid, and the acid-insoluble radioactivity which was collected on a glass fiber filter was counted in a liquid scintillation spectrometer.

Systems	[ $^{14}\text{C}$ ] Leucine released (cpm $\times 10^{-8}$ )
Control (unincubated)	0.22
Control (incubated)	3.19
+ Enomycin 2 nM	3.07
+ Enomycin 5 nM	2.65
+ Sparsomycin 50 $\mu\text{M}$	0.38

suggesting that chain initiation becomes limiting in the presence of enomycin (5 nM). This idea was further supported by the results of the following experiments (Table 1). The lysate which contained [ $^{14}\text{C}$ ]leucine-labeled nascent peptides on the polyribosomes was incubated for chain elongation and release from the ribosomes. Enomycin (5 nM) showed only a weak inhibition of the release of completed polypeptides. Sparsomycin, a potent inhibitor of chain elongation, strongly inhibited it. Thus it may be concluded that enomycin at less than 5 nM is a preferential inhibitor of chain initiation.

#### Effect of Enomycin on Steps of Chain Initiation

Initiation of protein synthesis in mammalian cells involves two distinct steps: (1) formation of the 40S ribosomal subunit-Met-tRNA<sub>f</sub> complex and (2) joining of the complex with mRNA and the 60S subunit to form the 80S initiation complex. The formation of the complexes may be conveniently assayed by incubation of the lysate system with [ $^{35}\text{S}$ ]methionine<sup>11-15</sup>. To investigate the effect of enomycin on these initiation steps the lysate system was incubated with the labeled methionine and the antibiotic, and the complexes formed

Fig. 3. Effect of enomycin on formation of the initiation complexes

The systems (220  $\mu\text{l}$ ) containing [ $^{35}\text{S}$ ]methionine (23  $\mu\text{Ci/ml}$ , 50 Ci/mmol) were incubated for 4 minutes (A) and 9 minutes (B). Enomycin (5 nM) was added at 4 minutes and the mixture was incubated for additional 5 minutes (C). The reaction was terminated by chilling and addition of 400  $\mu\text{l}$  of cold buffer A. A 500  $\mu\text{l}$  aliquot was layered on a sucrose gradient. Centrifugation was for 4.5 hours.

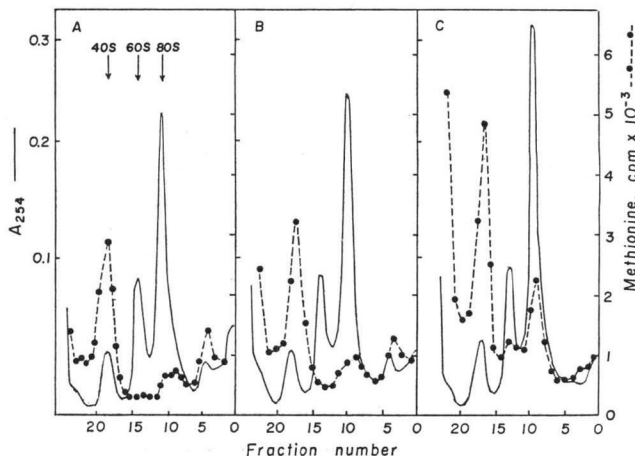


Fig. 4. The time course of accumulation of methionine radioactivity on the 40S subunits and the 80S ribosomes by enomycin. The systems (220  $\mu$ l) were incubated with [ $^{35}$ S]-methionine (46  $\mu$ Ci/ml, 75 Ci/mole) in the presence or absence of enomycin (5 nM). To one system which had been incubated for 6 minutes with enomycin, puromycin (0.5 mM) was added and the mixture was further incubated for 3 minutes. After the incubation the mixtures were treated as described in the legend to Fig. 3. To quantitate the radioactivity associated with the 40S subunits and the 80S ribosomes, radioactive counts in the individual fractions (usually five in number) were totaled and plotted with time. An aliquot (25  $\mu$ l) of the system was also taken for the assay of protein synthesis. (A) 40S subunit; (B) 80S ribosome; (C) protein synthesis.

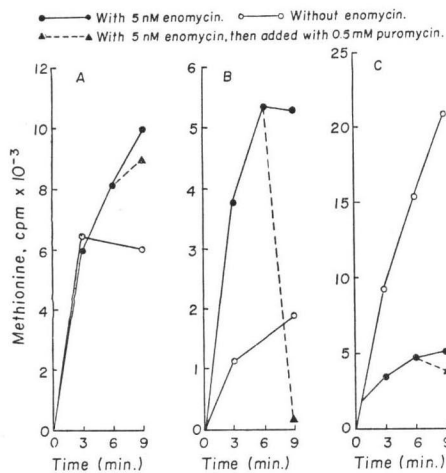
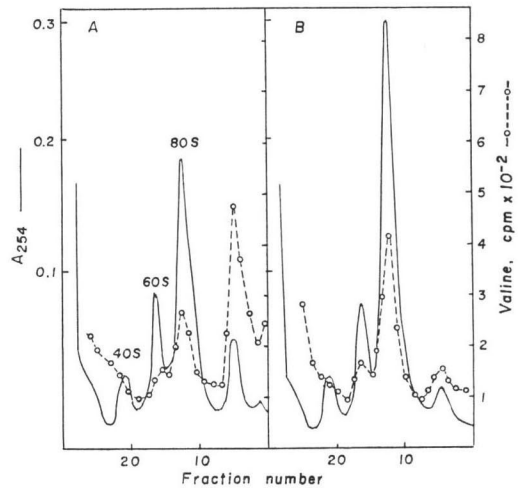


Fig. 5. Effect of enomycin on Val-tRNA binding to ribosomes. The systems (220  $\mu$ l) containing [ $^3$ H]Val-tRNA ( $5 \times 10^5$  cpm, 4.8 Ci/mole) were incubated for 5 minutes without (A) or with (B) enomycin (5 nM). After the incubation, the mixtures were treated as described in the legend to Fig. 3.



were analyzed in a sucrose gradient. As shown in Fig. 3, enomycin caused an increase in the 40S complex as well as the 80S complex. The time course of this increase indicated that methionine radioactivity accumulated more rapidly on the 80S ribosomes than on the 40S subunits (Fig. 4). The accumulated radioactivity on the ribosomes was

identified as [ $^{35}$ S]methionine bound as Met-tRNA by cellulose thin-layer chromatography (data not shown). The accumulated label on the 80S ribosomes was completely released from the ribosomes when the inhibited system was incubated with puromycin (Fig. 4B), while that on the 40S subunits was not (Fig. 4A). In an assay of the trinucleotide codon AUG-promoted joining of the preformed 40S complex with the 60S subunit to form the 80S complex<sup>5, 15, 16</sup>, enomycin showed no inhibition of the reaction (not shown). Thus enomycin does not inhibit the two steps of initiation but rather causes the increase in the initiation complex.

When the lysate was incubated with [ $^3$ H]Val-tRNA (the second amino acid of nascent globin chains) the bound radioactivity on the 80S ribosomes was slightly increased by enomycin, while that on the ribosome dimer was markedly reduced (Fig. 5). The results suggest that Val-tRNA binding to the 80S initiation complex is not inhibited, although the incorporation of the amino acid into polypeptides is blocked.

#### Inhibition of Initial Dipeptide Formation by Enomycin

Several inhibitors which are known to inhibit chain initiation allow synthesis of the initial

Fig. 6. Thin-layer chromatographic analysis of peptides synthesized

The systems (220  $\mu$ l) containing f[<sup>35</sup>S]Met-tRNA<sub>f</sub> ( $7 \times 10^5$  cpm) were incubated for 10 minutes without (A) or with (B) enomycin (3 nM), with pactamycin (2  $\mu$ M) (C), and with enomycin and pactamycin (D). For (D) pactamycin was added after the system was incubated for 2 minutes with enomycin. The front of the solvent was at 16 cm from the origin.

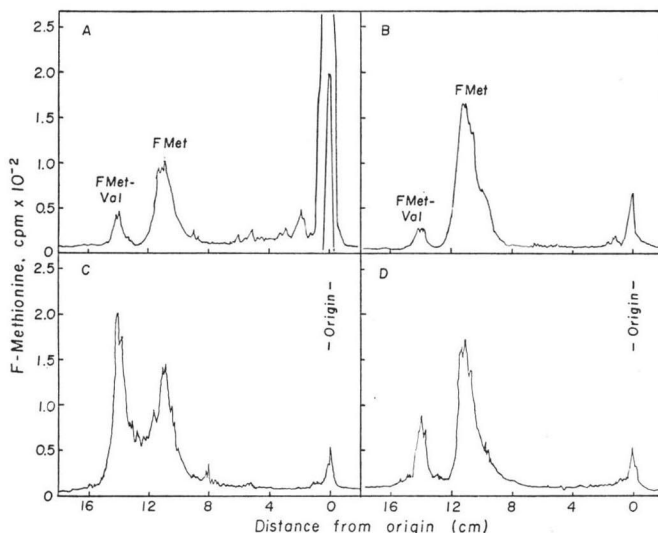


Table 2. Inhibition of pactamycin-induced accumulation of fMet-Val by enomycin

The systems were incubated as described in the legend to Fig. 6. After thin-layer chromatography, the cellulose was scraped from the spot of fMet-Val marker and the powder was extracted with 1 ml of 0.1 M NaOH at 34°C for 10 minutes. A 0.7 ml aliquot of the extract was mixed with 2 ml of Soluene 350 (Packard) and counted in a liquid scintillation spectrometer.

	fMet-Val radioactivity (cpm)
+ Pactamycin (2 $\mu$ M)	1,840
+ Enomycin (2.5 nM) and pactamycin	1,230
+ Enomycin (5.0 nM) and pactamycin	990
+ Enomycin (10 nM) and pactamycin	730
- Pactamycin	310
+ Enomycin (5 nM)	130

dipeptide of globin chains but cause accumulation of it on ribosomes in the lysate system<sup>5,17,18</sup>). The antibiotic pactamycin is one of them<sup>17,18</sup>). Therefore to determine the effect of enomycin on initial dipeptide synthesis, pactamycin-induced accumulation of fMet-Val dipeptide from fMet-tRNA<sub>f</sub> was estimated by cellulose thin-layer chromatography (Fig. 6). The radioactivity at the origin which quantitatively represented polypeptides synthesized was severely inhibited by enomycin and pactamycin. Moreover increase of fMet by enomycin and fMet-Val by pactamycin were evident. This pactamycin-induced fMet-Val increase was inhibited by enomycin when the system was preincubated with the antibiotic before pactamycin was added (Fig. 6D). The rates of inhibition were dependent on the amounts of the antibiotic added (Table 2),

and the small synthesis of the dipeptide in the system without pactamycin was also significantly decreased by enomycin (5 nM). When the lysate system was incubated with both the drugs at the same time little inhibition was shown, suggesting that pactamycin may work more rapidly than enomycin.

### Discussion

As demonstrated in this paper enomycin at less than 5 nM preferentially inhibits an initial process of protein synthesis in the lysate system without significant effect on chain elongation. YAMAKI *et al.*<sup>19)</sup> recently reported the similar selective inhibition of globin synthesis initiation by the polypeptide antibiotic phenomycin. We previously showed, however, that enomycin at more than 10 nM inhibited protein synthesis in rat liver cell-free system primarily by an effect on chain elongation<sup>2)</sup>. Indeed with enomycin at 50 nM in the lysate system polyribosome disaggregation was severely retarded (unpublished results). Thus at the higher concentrations the antibiotic acts as an inhibitor of chain elongation.

Protein synthesis initiation is defined as a process which involves formation of the 40S subunit-Met-tRNA<sub>f</sub> complex and association of the complex with mRNA and the 60S subunit to form the 80S complex<sup>20)</sup>. Enomycin did not inhibit these processes but rather promoted them. The kinetics of accumulation of the 80S and the 40S complexes suggests that the accumulation of the 80S complex may cause the accumulation of the 40S complex. In this sense enomycin even at the lower concentrations is not an inhibitor of chain initiation. Moreover the 80S complex could react with puromycin, and methionine radioactivity was released under the inhibited conditions, indicating that Met-tRNA<sub>f</sub> is properly bound in a puromycin-reactive state (*i.e.*, in the P-site) and peptidyltransferase activity is not affected.

The next step in protein synthesis is binding of Val-tRNA, the second amino acid of nascent globin chains, and this was not inhibited by enomycin. However the initial dipeptide accumulation which was induced by pactamycin was inhibited by enomycin. The results indicate that the dipeptide bond formation is inhibited or alternatively the ribosome bound dipeptide (dipeptidyl-tRNA) is destabilized and released by enomycin. When the lysate containing nascent peptides on the polyribosomes was incubated with enomycin (100 nM), little release of the label was shown (unpublished results), indicating that the latter possibility may be excluded. Therefore the block in initial dipeptide bond formation could result from an impaired binding of Val-tRNA so that it is unreactive in peptide bond synthesis. There are two states of Val-tRNA binding; one is unreactive and the other reactive in peptide bond formation<sup>21)</sup>; transition to the reactive state (termed an accommodation step) requires GTP hydrolysis<sup>22-25)</sup>. Moreover YOKOSAWA *et al.*<sup>24)</sup> have reported that GTP hydrolysis is required for release of Tu from ribosomes as Tu-GDP, which is a prerequisite for peptide bond formation. Enomycin may inhibit this step by its preferential affinity for initiating ribosomes or ribosomal subunits. Further study is needed using partial reactions of chain elongation to make a direct evaluation. At present it is not clear whether enomycin selectively inhibits the initial dipeptide synthesis or also inhibits the synthesis of some oligopeptides.

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