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Initiation of Hemoglobin Synthesis. Specific Inhibition by Antibiotics and Bacteriophage Ribonucleic Acid*

Harvey F. Lodish, David Housman, and Marion Jacobsen

ABSTRACT: In extracts of rabbit reticulocytes, both globin chains are initiated by a specific methionyl transfer RNA (Met-tRNA$_f^{Met}$). Normally, the amino-terminal methionyl residue is removed from the nascent chains. However, if the methionine amino group on the tRNA is blocked by a formyl residue (fMet-tRNA$_f^{Met}$) the modified tRNA initiates globin synthesis normally, but the amino terminal fMet residue is not removed. Hence, incorporation into globin of radioactivity from f[^{35}S]Met-tRNA$_f^{Met}$ provides a rapid assay for initiation of globin synthesis. Here we show that, at the minimum concentration necessary to completely block incorporation from fMet-tRNA$_f^{Met}$, both pactamycin and aurintricarboxylic acid specifically block initiation of globin chains, but do not affect the rate of completion of the existing nascent chains. There is incorporation of other radioactive amino acids, specific for the internal positions of globin, just sufficient to complete the existing chains, and all polyribosomes are converted to monoribosomes with release of a complete globin chain. At higher concentrations both drugs do affect some reaction in polypeptide chain elongation, as can be seen both by reduced incorporation of radioactivity into the nascent chains, and by reduced movement of ribosomes along the mRNA. RNA from bacteriophage f2 also specifically blocks initiation of globin chains. When f2 RNA is treated with formaldehyde, to relax partially its secondary structure, it becomes a considerably more potent inhibitor of globin initiation, and the treated RNA binds to reticulocyte ribosomes. By contrast, the antibiotics cycloheximide and emetine do not specifically inhibit initiation of globin chains; over a wide concentration range tested the primary inhibitory effect was on propagation of the nascent chain.

A chemical which specifically blocks initiation of protein biosynthesis in eucaryotic cells is invaluable for many types of studies on the mechanism and regulation of gene expression. Three compounds have been reported to possess this specificity: aurintricarboxylic acid (Grollman and Stewart, 1968) and the antibiotics cycloheximide (Lin *et al.*, 1966) and pactamycin (Colombo *et al.*, 1966; Cohen *et al.*, 1969; MacDonald and Goldberg, 1970). Under certain conditions, however, all

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of these chemicals appear to inhibit as well other steps in the propagation of the polypeptide chain: cycloheximide (McKeehan and Hardesty, 1969), aurintricarboxylic acid (Siegelman, 1970), and pactamycin (MacDonald and Goldberg, 1970).

Part of the difficulty in the study and application of these drugs has been the lack of any rapid assay for initiation of protein synthesis in extracts of eucaryotic cells. In particular, most eucaryotic cell-free systems employed, such as the reticulocyte, are not dependent on addition of exogenous natural mRNA, and the endogenous mRNA is bound to polysomes. Hence it is difficult to dissociate effects on the completion of existing nascent chains from those on the initiation of new chains. Most workers have used as criteria for specific inhibition of chain initiation either the disappearance of polyribosomes and appearance of monosomes and/or subunits or the inhibition of incorporation of radioactive amino acids into the amino terminus of the finished protein. Certain synthetic polynucleotides, as poly(U) and poly(A), apparently also block initiation of hemoglobin synthesis, probably by interfering with attachment of mRNA to the ribosome (Hardesty *et al.*, 1963).

Recently, Brown and Smith (1970) and Smith and Marcker (1970) proposed that one of two methionyl-tRNAs found in eucaryotic cells, that which can be formylated by bacterial enzymes (Met-tRNA$_f^{Met}$),[1] is the initiator for protein synthesis. Hemoglobin (Wilson and Dintzis, 1970; Jackson and Hunter, 1970; Housman *et al.*, 1970) and other eucaryotic proteins (yeast cytochrome *c*, Sherman *et al.* (1970); protamine, Wiegle and Dixon (1970)) indeed are initiated by Met-tRNA$_f$. The other methionyl-tRNA (Met-tRNA$_M^{Met}$), in contrast, transfers radioactivity exclusively into the internal positions of eucaryotic proteins.

In rabbit reticulocytes, the amino-terminal Met is removed from the nascent hemoglobin polypeptide exposing the penultimate valine as amino terminus (Wilson and Dintzis, 1970; Housman *et al.*, 1970). We showed previously that if the methionine amino group of yeast Met-tRNA$_f^{Met}$ is blocked by a formyl residue (fMet-tRNA$_f^{Met}$), it will initiate hemoglobin synthesis as efficiently as the natural nonformylated derivative. However, the amino-terminal fMet residue is not removed; thus incorporation of radioactivity into protein from f[^{35}S]Met-tRNA$_f^{Met}$ is a direct measure of *in vitro* initiation of hemoglobin synthesis.

In this paper we show that, at low concentrations, both ATA and pactamycin do specifically block initiation of hemoglobin synthesis: there is no incorporation from f[^{35}S]Met-tRNA$_f^{Met}$; incorporation from [^{35}S]Met-tRNA$_M^{Met}$ or [^{14}C]-lysine is normal for 1–2 min (at 25°) and then ceases. That this synthesis represents only completion of existing chains is shown by the rapid and complete conversion of polyribosomes to monosomes and subunits. At higher concentrations, in contrast, both drugs block some step in the elongation of peptide chains, for both completion of the nascent chains and ribosome movement along the mRNA are blocked. Cycloheximide and emetine (Grollman, 1966) by contrast, over a wide range of concentrations, do not appear to preferentially block initiation of hemoglobin chains.

In bacterial extracts, the three proteins coded by bacteriophage f2 RNA also are initiated by fMet-tRNA (Adams and Capecchi, 1966; Webster *et al.*, 1966; Viñuela *et al.*, 1967; Lodish, 1968, 1969; Lodish and Robertson, 1969). Hence it is of interest that in rabbit reticulocyte lysate f2 RNA also specifically blocks initiation of hemoglobin synthesis. When f2 RNA is treated with formaldehyde at 37°, there is a partial relaxation of the RNA secondary structure and new sites on the RNA are exposed which can initiate bacterial protein synthesis (Boedtker, 1967; Lodish, 1970). The treated RNA inhibits hemoglobin initiation much better than normal f2 RNA; the treated RNA also binds to reticulocyte 80S ribosomes. However, we have no evidence that either the normal or formaldehyde-treated f2 RNA is translated by reticulocyte extracts.

Materials and Methods

Protein Synthesis in Reticulocyte Extracts. The standard reaction mixture contained, per ml, 0.62 ml of reticulocyte lysate, 0.92 μmole of ATP, 0.19 μmole of GTP, 50 μmoles of KCl, 5 μmoles of 2-mercaptoethanol, 11 μmoles of creatine phosphate, 1 mg of creatine phosphokinase, 25 μmoles of Hepes (pH 7.0), 2.0 μmoles of magnesium acetate, and 34 μg of haemin. Preparation of the reticulocyte extract was described in detail previously (Housman *et al.*, 1970). Reactions containing radioactive Met-tRNAs also contained nonradioactive methionine (1.2 μmoles) and 19 other nonradioactive amino acids (0.1 μmole each) and either 9×10^5 cpm of f[^{35}S]Met-tRNA$_f^{Met}$ (48,000 cpm/μg of tRNA), 7×10^5 cpm of [^{35}S]Met-tRNA$_f^{Met}$ (30,000 cpm/μg of tRNA), or 8×10^5 cpm of [^{35}S]Met-tRNA$_M^{Met}$ (4500 cpm/μg of tRNA). The [^{35}S]methionine had a specific activity of about 6000 mCi/mmole, and the above figures are based on a counting efficiency of 22%.

Reactions containing [^{14}C]lysine contained, per ml, [^{14}C]-lysine (246 mCi/mmole; 4 μCi/ml) and 19 other nonradioactive amino acids (0.1 μmole).

Reaction mixtures, generally 0.06 ml, were incubated at 25°. Aliquots of either 10 or 15 μl, as indicated in each figure, were taken at intervals into 0.5 ml of ice-cold water, and immediately 0.5 ml of 0.2 M KOH was added. After incubation at 37° for 10 min trichloroacetic acid was added to 5%. The precipitate was collected on fiber glass filters (Whatman GF/C, 2.4 cm diameter), washed five times with 5 ml of 5% trichloroacetic acid solution, dried, and counted in a low-background counter at 22% efficiency.

Sucrose Density Gradient Centrifugation Analyses. Aliquots, generally 0.25–0.36 ml, of the cell-free reactions were chilled rapidly in ice. One milliliter of buffer A (0.025 M Hepes (pH 7.0)–0.05 M KCl–0.002 M magnesium acetate) was added, and the solution was layered on a 36-ml linear sucrose gradient (15–50% w/v) in buffer A. Centrifugation was for 6.5 hr at 26,500 rpm in the SW27 rotor of the Beckman L-3 or L2-65B ultracentrifuge; temperature was maintained between 2 and 4°.

The gradient was pumped from the bottom through a 5-mm flow cell in a Gilford spectrophotometer, and the absorbance at 260 nm was continously recorded. When the gradients contained radioactive amino acids, approximately 1.0-ml fractions were collected. To them was added KOH to 0.1 M; incubation, precipitation, and counting were done as in the previous section. If samples did not yield a visible precipitate after addition of trichloroacetic acid, then 0.5 mg of bovine serum albumin was added as carrier. Because of the large amount of protein in the top fractions, only 0.1- and 0.2-ml aliquots were precipitated and counted.

[1] Nonstandard abbreviations used are: CYC, cycloheximide; EM, emetine; ATA, aurintricarboxylic acid; fMet, *N*-formylmethionine; tRNA^{Met}, the species of methionine-acceptor tRNA which can be formylated by *Escherichia coli* transformylase; tRNA$_M^{Met}$, the species of methionine-acceptor tRNA which cannot be formylated; Hepes,

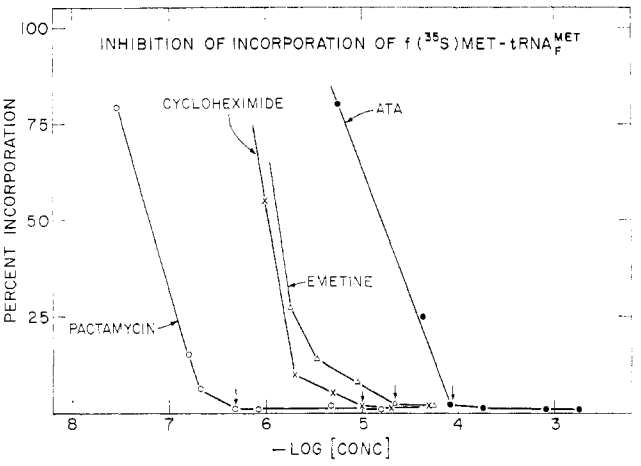

FIGURE 1: Inhibition of incorporation of f[^{35}S]Met-tRNA$_F$ by drugs. Reactions (60 μl) contained f[^{35}S]Met-tRNA$_f^{Met}$. At each concentration of drug a complete kinetic experiment was done; aliquots of 10 μl were taken after 0-, 5-, 10-, 20-, and 30-min incubation. In all cases incorporation was proportional to time of incubation for at least 20 min. Plotted here are the results from the 10-min samples; results from the 5- or 20-min samples are not significantly different ($\pm$15%). Each drug was assayed in a separate experiment. The incorporation after 10-min incubation of the control samples was between 440 and 560 cpm per 10-μl aliquot; zero-time backgrounds of 14–22 cpm have been subtracted. The vertical arrows represent the minimum concentration needed to completely block incorporation.

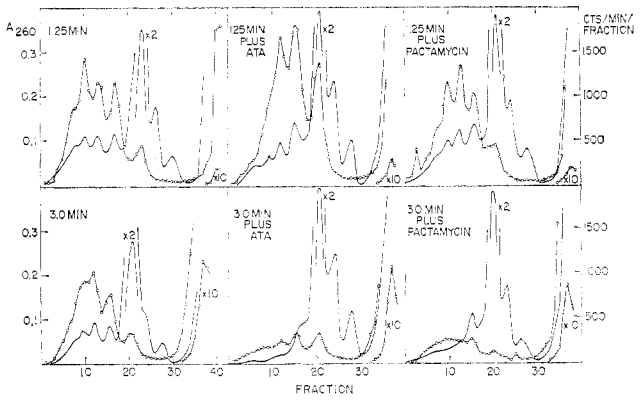

FIGURE 3: Reactions containing [^{35}S]Met-tRNA$_M^{Met}$ and, where indicated, either ATA (10^{-4} M) or pactamycin (8×10^{-7} M) were described in the legend to Figure 2. Samples (0.30 ml) were removed after 1.25- and 3.0-min incubation, chilled to 0°, and analyzed on 15–50% sucrose gradients as described in Materials and Methods. From the data in Figure 2 we calculate that the following amount of polypeptide ratioactivity was placed on the gradients. Control: 1.25 min, 21,500 cpm; and 3.0 min, 60,500 cpm. ATA: 1.25 min, 25,200 cpm; and 3.0 min, 35,000 cpm., Pactamycin: 1.25 min, 21,000 cpm; 3.0 min, 29,000 cpm. A tabulation of radioactivity in the different gradient fractions is in Table I. Centrifugation is from right to left. In the control 1.25-min sample monoribosomes (80 S) are in fraction 23; the large (60 S) and small subunits (40 S) are in fractions 26 and 30, respectively. Disomes are in fraction 16.

Binding of [^{32}P]f2 RNA. [^{32}P]f2 RNA (3×10^7 cpm/mg) was prepared as described previously (Lodish *et al.*, 1965) except that, in the purification of the phage, precipitation by ammonium sulfate was replaced by coacervation with polyethylene glycol (Leberman, 1966; Yamamoto *et al.*, 1970). Cell-free reactions (0.12 ml) were described in the previous section, except that they contained 0.1 μmole/ml of all 20 nonradioactive amino acids, and 6×10^5 cpm of [^{32}P]f2 RNA or [^{32}P]RNA which was treated with formaldehyde. Incubation was at 25° for 5 min; the reactions were cooled and layered on a 36-ml sucrose gradient as described above. Centrifugation was for 16.5 hr at 21,000 rpm. Aliquots of 0.1 ml of the 1.0-ml fractions were placed on fiber glass filter pads, dried, and counted in a Beckman LS-250 scintillation counter.

Bacteriophage f2 RNA. Isolation of f2 RNA and treatment of the RNA with 1 M formaldehyde were detailed previously (Lodish, 1970).

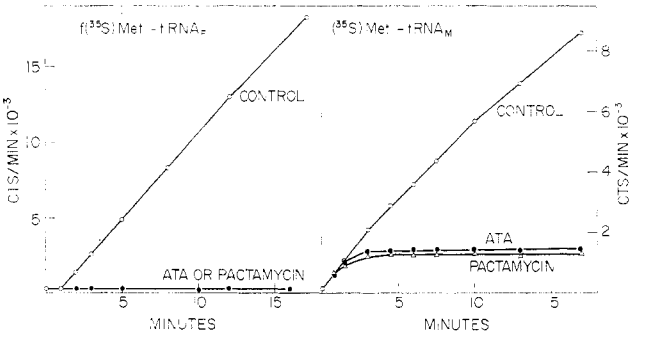

FIGURE 2: Protein synthesis in the presence of ATA or pactamycin. Reactions (840 μl) contained f[^{35}S]Met-tRNA$_f^{Met}$ (left) or [^{35}S]-Met-tRNA$_M^{Met}$ (right) and, where indicated, ATA (10^{-4} M) or pactamycin (8×10^{-7} M); 10-μl aliquots were counted. The remainder of the [^{35}S]Met-tRNA$_M^{Met}$ reactions were used for the polyribosome studies in Figure 3.

tRNA. The methionine tRNAs, tRNA$_f^{Met}$ and tRNA$_M^{Met}$, were purified from yeast by Dr. U. L. RajBhandary, following the procedure of RajBhandary and Ghosh (1969). The former tRNA was 90% pure, and the latter 10%; neither preparation was contaminated by detectable amounts of the other tRNA species. *Escherichia coli* enzymes were used to charge the tRNAs with [^{35}S]methionine and to formylate [^{35}S]Met-tRNA$_f^{Met}$ with formyltetrahydrofolic acid (RajBhandary and Ghosh, 1969). As measured by the amounts of [^{35}S]methionyladenosine and formyl[^{35}S]methionyladenosine after RNase A digestion and paper ionophoresis at pH 3.5, [^{35}S]Met-tRNA$_f^{Met}$ and [^{35}S]Met-tRNA$_M^{Met}$ contained no detectable formylated species, while f[^{35}S]Met-tRNA$_f^{Met}$ contained 1–1.5% nonformylated species.

Materials. Cycloheximide was purchased from Nutritional Biochemicals, emetine from Mann Laboratories, and aurintricarboxylic acid (Aluminon lot 790040) from Fisher. Pactamycin was a generous gift of Dr. Irving Goldberg. [^{14}C]Lysine (246 mCi/mmole) was purchased from New England Nuclear Corp., as the the carrier-free [^{35}S]H_2SO_4 used to prepare [^{35}S]-methionine (5000–6000 mCi/mmole) by the procedure of Sanger *et al.* (1965). Creatine phosphate and creatine phosphokinase were obtained from Calbiochem.

Results

In crude lysates of rabbit reticulocytes, over 90% of the protein produced is hemoglobin. Incorporation into protein of radioactivity from [^{35}S]Met-tRNA$_f^{Met}$ is a measure of initiation of hemoglobin synthesis; formyl[^{35}S]methionine residues are incorporated only into the amino termini of α and β chains (Housman *et al.*, 1970). Radioactivity from [^{35}S]Met-tRNA$_M^{Met}$ or [^{14}C]lysine is incorporated only into the internal amino acids of hemoglobin; hence incorporation of these compounds

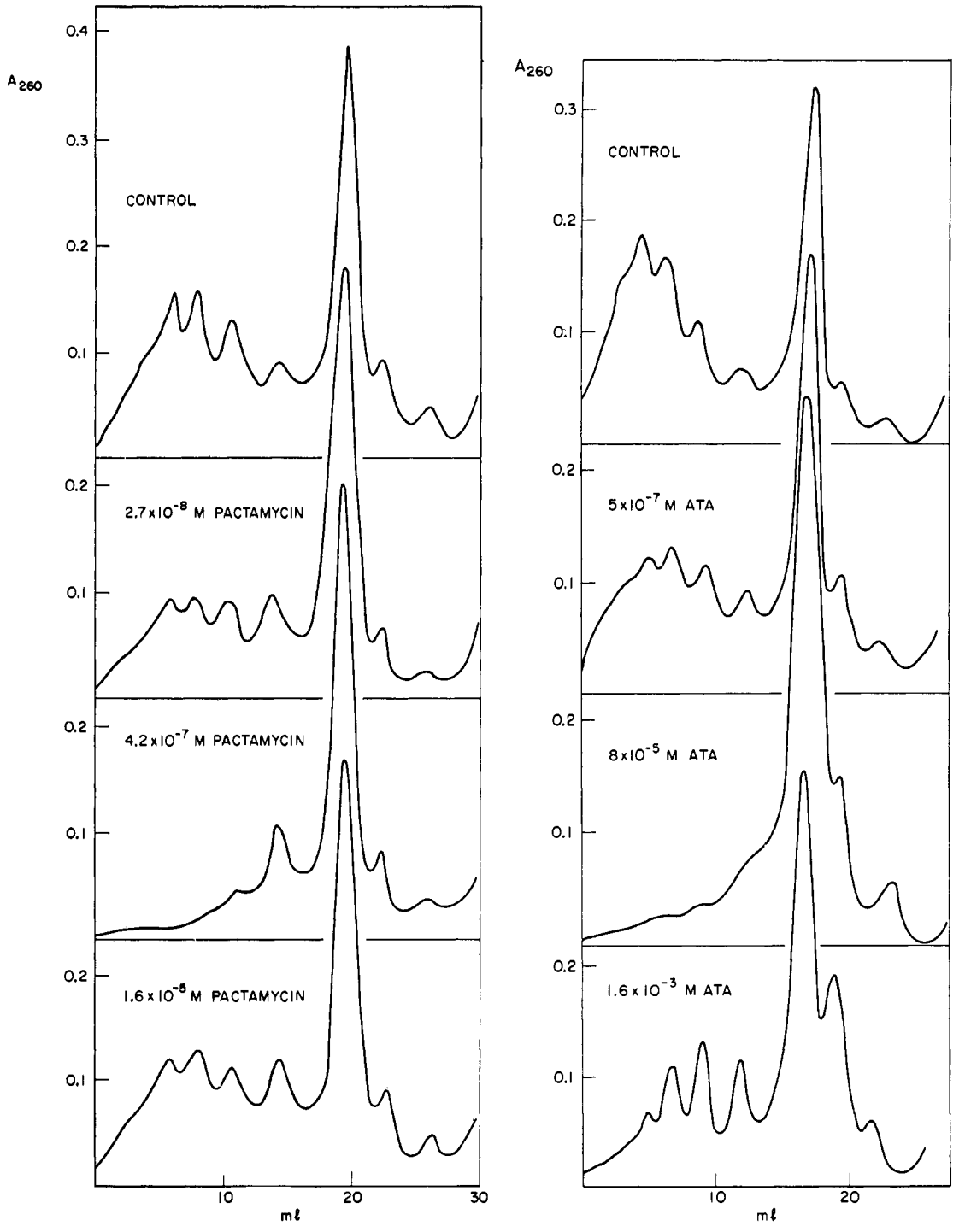

FIGURE 4: Polysome profiles in the presence of different concentrations of pactamycin (a, left) or ATA (b, right). Reactions (0.30 ml) were analyzed after incubation at 25° for 3.0 min.

represents completion both of polypeptides which are in the process of synthesis when the extract is prepared and of proteins which are initiated during the course of the *in vitro* synthesis.

To investigate the mechanism of action of a chemical we first determined the minimum concentration necessary to completely block *initiation* of hemoglobin chains, as measured by incorporation of f[^{35}S]Met-tRNA$_f^{Met}$. If the primary effect of the drug were on propagation of the polypeptide chain, or if the drug inhibits initiation and extension to the same extent, then we would expect this concentration to completely inhibit incorporation of internal amino acids as well. In contrast, if the drug specifically blocks initiation, then one would expect an incorporation of internal amino acids just sufficient to complete the existing nascent chains. Also, as the ribosomes are unable to initiate new chains, the polysomes should disappear as the ribosomes complete the nascent chains.

All of these experiments were done at 25°, in order to lengthen the time required to synthesize a single polypeptide chain.

ATA and Pactamycin. The minimum concentration of these drugs necessary to completely inhibit incorporation of f[^{35}S]-

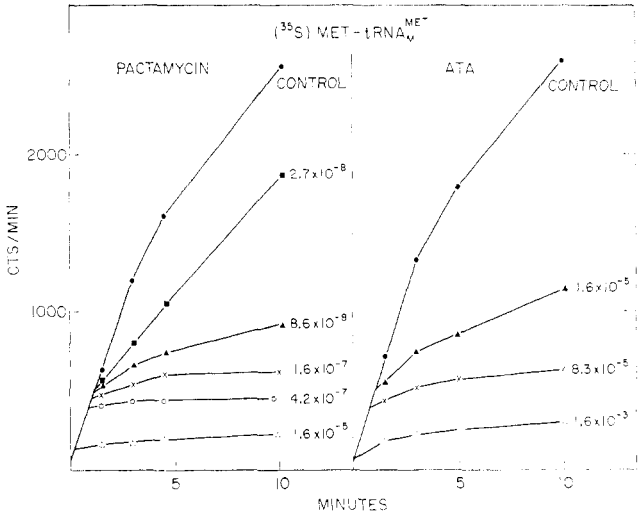

FIGURE 5: Effect of different concentrations of ATA or pactamycin on transfer of radioactivity from [^{35}S]Met-tRNA$_M^{Met}$ into protein. Concentrations of the drugs are in moles per liter; 15-μl aliquots are counted.

Met-tRNA$_f^{Met}$ is 8×10^{-5} and 5×10^{-7} M, respectively (Figure 1). Figure 2 shows that these concentrations specifically block initiation of hemoglobin synthesis. Incorporation from [^{35}S]Met-tRNA$_M^{Met}$ (Figure 2b) or [^{14}C]lysine or [^{14}C]leucine (data not shown) is normal for about 1–2 min and then ceases.

The conclusion from this experiment that ATA and pactamycin only permit completion of nascent hemoglobin chains is confirmed by the polysome studies in Figure 3. After an incubation at 1.25 min at 25°, the control sample consists of approximately equal numbers of di-, tri-, tetra-, and pentasomes. Virtually all of the [^{35}S]Met from Met-tRNA$_M^{Met}$ incorporated into polypeptide linkage is on the polyribosomes and relatively little is in released chains at the top of the gradient (Table I). After a 3 min incubation the polyribosome profile is virtually unchanged and now, as expected, a large portion of incorporated radioactivity is found in completed chains on the top of the gradient.

After a 1.25-min incubation of the samples treated with ATA or pactamycin, essentially the same amount of [^{35}S]Met from [^{35}S]Met-tRNA$_M^{Met}$ is found on the polyribosomes, and

TABLE I: Distribution of Radioactivity from [^{35}S]Met-tRNA$_M$.[a]

Time (min)	Addn (M)	Polysomes	Monosomes	Top	Total
1.25	0	13,760	1,780	4,600	20,140
	ATA (10^{-4})	16,140	4,020	6,880	27,040
	Pactamycin (8×10^{-7})	11,960	1,880	5,320	19,160
3.0	0	*9,600*	1,060	34,440	45,060
	ATA (10^{-4})	*3,020*	1,080	29,160	33,260
	Pactamycin (8×10^{-7})	*3,180*	460	23,060	26,700

[a] Tabulated are the total amounts of acid-precipitable radioactivity from the three regions of the sucrose gradients in Figure 3. The "top" regions includes all fractions sedimenting slower than monoribosomes.

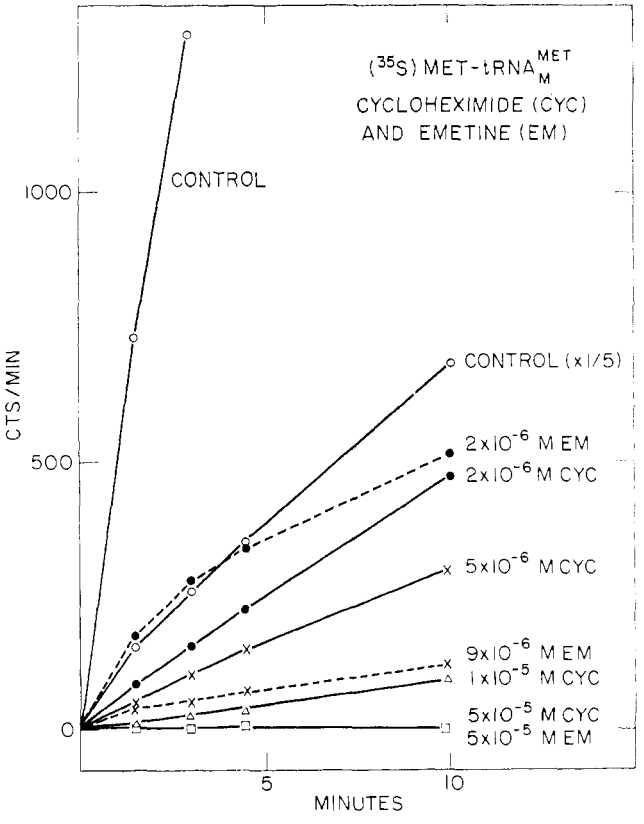

FIGURE 6: Effect of cycloheximide (CYC) and emetine (EM) on transfer of radioactivity from [^{35}S]Met-tRNA$_M^{Met}$ into protein.

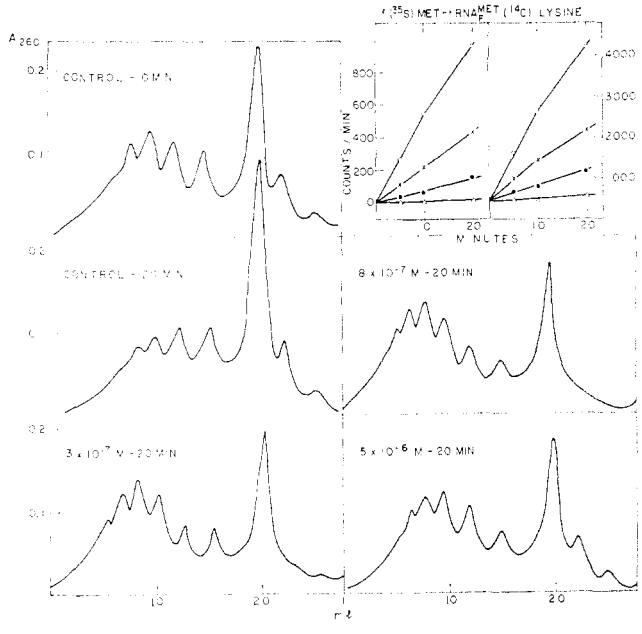

FIGURE 7: Polyribosome profile after 20-min incubation in the presence of different concentrations of cycloheximide. Insert: protein synthesis (8-μl aliquots) using [^{14}C]lysine or f[^{35}S]Met-tRNA$_f^{Met}$, measured on the same reactions as used for polysome studies. (⊙) Control, (×) 3×10^{-7} M cycloheximide, (●) 8×10^{-7} M cycloheximide, and (Δ) 5×10^{-6} M cycloheximide. The rabbit extract used was different from the one used in the experiments described in Figures 1, 6, and 8.

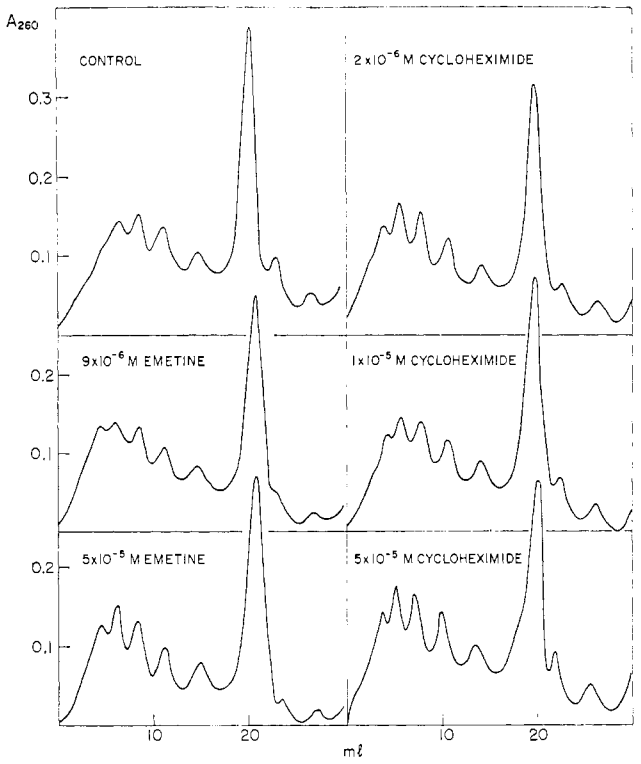

FIGURE 8: Polyribosome profiles after incubation of 3.5 min in the presence of cycloheximide or emetine.

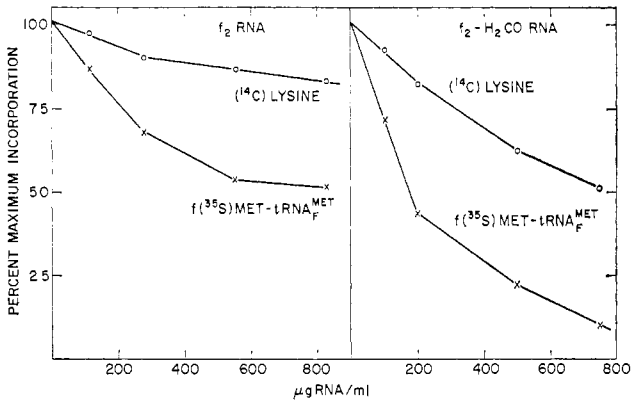

FIGURE 9: Effect of f2 RNA (left) or formaldehyde-treated f2 RNA (right) on reticulocyte protein synthesis. Again a complete kinetic experiment was performed and aliquots (10 μl) were taken after 0, 5, 10, 20, and 40 min. Incorporation in the control sample was linear for at least 20 min; data from the 5-min sample are plotted. Incorporation at 5 min in the absence of viral RNA was [^{14}C]lysine, 1250 cpm/10 μl; f[^{35}S]Met-tRNA$_f^{Met}$, 983 cpm; zero-time backgrounds have been subtracted; [^{14}C]lysine, 28 cpm; f[^{35}S]Met, 41 cpm.

again, little in released chains. However, there are relatively many more disomes and fewer of the larger polyribosomes, suggesting partial detachment of ribosomes from the mRNA. After 3-min incubation virtually all of the polyribosomes have disappeared, resulting in only a small amount of disomes and a proportional increase in the amount of monoribosomes. Hence all of the ribosomes in polysomes can complete an existing nascent hemoglobin chain, but cannot reinitiate one. Furthermore, essentially all of the radioactivity found on polysomes after 1.25-min incubation has disappeared from the polysomes, and is found at the top of the gradient. Analysis on carboxymethylcellulose columns showed that indeed over 90% of the released material is completed α and β chains.

Since both ATA and pactamycin only permit completion of nascent chains, we can estimate, from Figures 2 and 3, that at 25° it takes approximately 120 sec to synthesize a complete hemoglobin molecule. This is in fair agreement with the estimate of Hunt *et al.* (1969) of 60–90 sec obtained from quite different experiments on whole cells.

We emphasize that the results obtained with both ATA and pactamycin are critically dependent on drug concentration. A concentration of pactamycin (2.7×10^{-8} M) $^1/_{20}$th of that used in Figures 2 and 3 only partially blocks incorporation of the initiator f[^{35}S]Met-tRNA$_f^{Met}$ (Figure 1), and as a consequence run-off of polyribosomes is greatly reduced (Figure 4a). On the other hand, a 40-fold higher concentration (1.6×10^{-5} M) of pactamycin affects some reaction in polypeptide chain elongation as well. This is seen in the reduced incorporation of [^{35}S]Met-tRNA$_M^{Met}$ (Figure 5) compared to 4×10^{-7} M pactamycin, a result which suggests that the higher concentrations inhibits the rate of completion of the existing nascent chains. Also after 3-min incubation with this concentration there is a marked reduction in the extent of disappearance of polyribosomes (Figure 4a).

Similar results were obtained with ATA; at 1.6×10^{-3} M, 20 times the minimum concentration necessary to block incorporation of f[^{35}S]Met-tRNA$_f^{Met}$, there is a marked reduction of incorporation of [^{35}S]Met-tRNA$_M^{Met}$ (Figure 5) and also only a slight disappearance of polyribosomes (Figure 4b). Hence, ATA at higher concentrations also blocks some stage in elongation of nascent polypeptides.

Cycloheximide and Emetine. Quite different results were obtained with both cycloheximide and emetine. At all concentrations inhibition of incorporation from f[^{35}S]Met-tRNA$_f^{Met}$ and [^{35}S]Met-tRNA$_M^{Met}$ is virtually the same. At the lowest concentration which completely blocks incorporation from the initiator f[^{35}S]Met-tRNA$_f^{Met}$ there is also no incorporation into the internal positions of hemoglobin (Figures 6 and 7). Both drugs inhibit incorporation from [^{14}C]lysine, [^{35}S]Met-tRNA$_M^{Met}$, and f[^{35}S]Met-tRNA$_f^{Met}$ to the same extent (Figures 1, 6 and 7 (insert)). These experiments suggest that the principal effect of both drugs is on propagation of the polypeptide chain. Confirming this, Figure 8 shows that, over a concentration range of both drugs, there is no alteration of the polyribosome profile after a 3 min incubation. Even when the incubation time is increased to 20 min, there is no change in the polyribosome profile in the presence of a concentration of cycloheximide which blocks protein synthesis 99%, measured by incorporation of either [^{14}C]-lysine or f[^{35}S]Met-tRNA$_f^{Met}$ (5×10^{-6} M in this extract) (Figure 7). After 20-min incubation in the absence of any drug there is a decreased amount of heavy polyribosomes, and an increased amount of monosomes; this indicates that normally some ribosomes have run off the mRNA and did not reinitiate. By contrast, concentrations of cycloheximide which inhibit protein synthesis 60 or 90% (3×10^{-7} and 8×10^{-7} M, respectively) result after this long incubation in an increased level of heavy polyribosomes and in the virtual disappearance of free ribosome subunits. Hence these concentrations of cycloheximide block attachment of ribosomes to mRNA less than they block polypeptide chain elongation. Hence, the primary effect of the drug is to inhibit movement of ribosomes along the mRNA; if cycloheximide does block initiation of globin chains, it does so at a higher concentration than needed to block elongation.

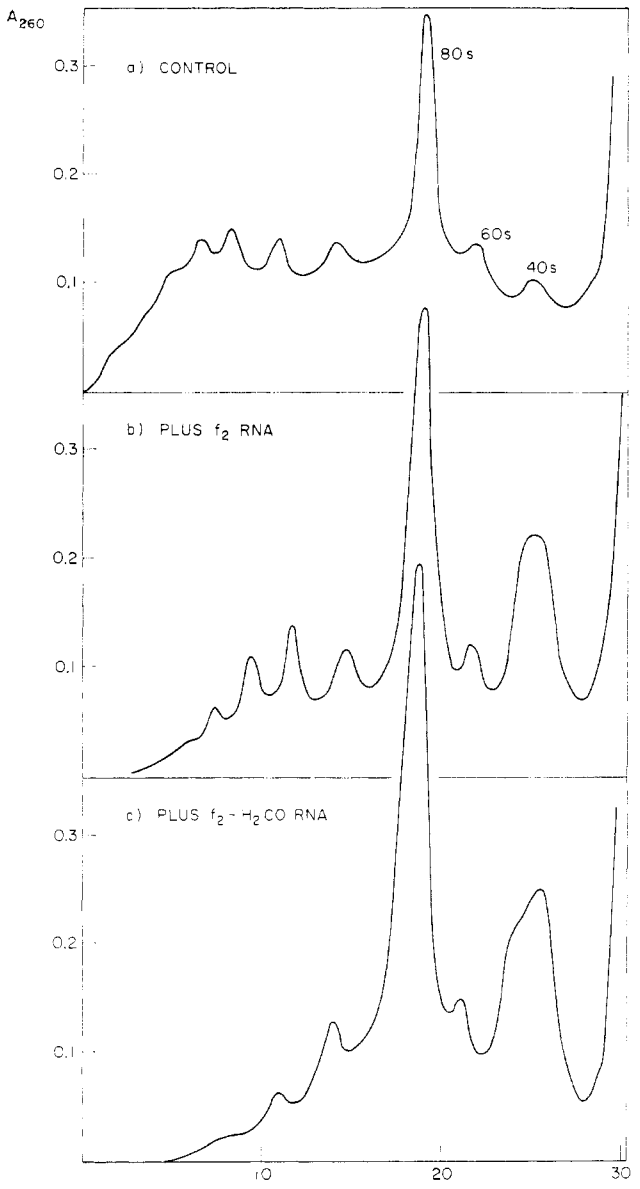

FIGURE 10: Polyribosome profile after 3-min incubation in the presence of 750 μg/ml of f2 RNA or formaldehyde-treated f2 RNA; 250-μl reactions were analyzed on 15–50% sucrose gradients. The large peak at 30 S (fraction 25) in b and c is mainly viral RNA.

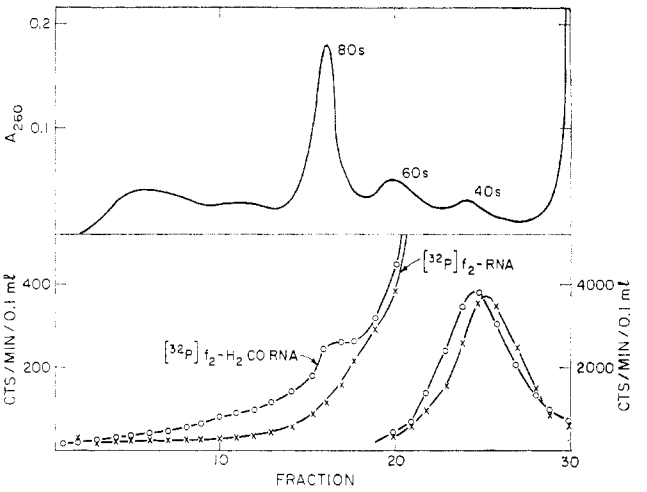

FIGURE 11: Cosedimentation of [^{32}P]f2 RNA with reticulocyte ribosomes. The experiment is detailed in Materials and Methods. The right-hand scale applies to fractions 20–30.

Inhibition of Initiation by f2 RNA. f2 RNA inhibits protein synthesis by reticulocyte cell-free systems. Incorporation of f[^{35}S]Met-tRNA$_f^{Met}$ is affected more than that of [^{14}C]lysine (Figure 9) suggesting that f2 RNA blocks preferentially initiation of hemoglobin synthesis. Even at the highest concentration (800 μg/ml) of RNA tested, however, inhibition was incomplete.

When f2 RNA is treated with 1 M formaldehyde at 37° for 10 min and then precipitated by ethanol to remove unreacted reagents, it directs protein synthesis in *E. coli* systems twice as efficiently as does untreated RNA (Lodish and Robertson, 1969; Lodish, 1970). The treatment enhances the rate of initiation of synthesis of f2 polymerase and maturation protein 4- and 20-fold, respectively. Furthermore, treated RNA initiates synthesis of at least three proteins which do not correspond to any f2 genes, a result implying that there are several nucleotides sequences in f2 RNA which can initiate protein synthesis in bacterial extracts but are normally prevented from doing so by the RNA structure (Lodish, 1970).

Figure 9 also shows that formaldehyde-treated f2 RNA is a better inhibitor of reticulocyte protein synthesis than is untreated RNA. Two types of experiments showed that the product made in the presence of f2 RNA or the formaldehyde-treated derivative is hemoglobin: (a) after removal of heme by precipitation with acid–acetone, over 80% of the labeled material chromatographs on a CMC column with authentic α and β chains; (b) trypsin digestion of product labeled with [^{35}S]Met-tRNA$_M^{Met}$ or f[^{35}S]Met-tRNA$_f^{Met}$, followed by ionophoresis at pH 3.5, showed that over 90% of the radioactivity migrated with the corresponding authentic peptides from the α and β chains (Housman *et al.*, 1970). The conclusion from Figure 9 that treated RNA preferentially blocks initiation of hemoglobin synthesis is supported by analysis of polyribosomes (Figure 10); treated RNA causes run-off of polyribosomes at least as quickly (3 min at 25°) as does ATA or pactamycin at optimal concentrations. Partial run-off of polysomes is observed in lysates treated with normal f2 RNA (Figure 10).

By analysis on sucrose gradients, [^{32}P]f2 RNA does not bind detectably to reticulocyte 80S ribosomes (Figure 11). In contrast, RNA treated with formaldehyde does bind to monoribosomes, and also a small amount sediments with the polyribosomes (Figure 11). This binding, which must be stable enough to withstand a 16-hr centrifugation, is equivalent to about 1 f2 RNA molecule/20 ribosomes. While this result suggests that formaldehyde-treated RNA is translated in this system, preliminary experiments using published techniques (Lodish, 1968–1970; Lodish and Robertson, 1969) have thus far failed to identify any f2-specific products.

[^{32}P]f2 RNA, whether treated with formaldehyde or not, is very stable in reticulocyte lysates. After incubation at 25° for 30 min in the standard reaction mixture, there was no loss of acid-precipitable material (±5%). No degradation of either RNA was observed when analyzed by sucrose gradient centrifugation; after incubation at 25° for 5 min the reaction was stopped by addition of sodium dodecyl sulfate to 0.5%, and the material layered on a 13-ml gradient (5–20% sucrose) (0.005 M Hepes (pH 7.0), 0.10 M NaCl, and 0.5% SDS) and centrifuged at 20°. Over 90% of the radioactivity sedimented

in a single peak, identical with that of unincubated RNA, at about 27 S.

Discussion

Initiation by f[^{35}S]Met-tRNA$_f^{Met}$. In these experiments we used yeast cytoplasmic tRNA$_f^{Met}$, charged and formylated by *E. coli* enzymes, to initiate hemoglobin synthesis. For the following reasons we believe that incorporation into hemoglobin of formyl[^{35}S]methionine from f[^{35}S]Met-tRNA$_f^{Met}$ is a valid measure of initiation of hemoglobin synthesis, and hence can be used to study the effects of inhibitors. (1) Transfer into protein of radioactivity from f[^{35}S]Met-tRNA$_f^{Met}$ is 60% that from [^{35}S]Met-tRNA$_M^{Met}$ (Housman *et al.*, 1970), a result suggesting that at least half the hemoglobin chains synthesized *in vitro* are initiated by the f[^{35}S]Met-tRNA$_f^{Met}$. However, it is difficult to make a more direct determination of the fraction of chains initiated *in vitro* by the added fMet-tRNA$_f^{Met}$. Our reticulocyte extracts also contain endogenous tRNA$_f$ charged with nonradioactive methionine which apparently initiates the other globin chains. (2) fMet-tRNA$_f^{Met}$ initiates globin synthesis as efficiently as does the natural initiator Met-tRNA$_f^{Met}$, for addition of a fourfold excess of unlabeled yeast Met-tRNA$_f^{Met}$ inhibits transfer from f[^{35}S]Met-tRNA$_f^{Met}$ by 80% (Housman *et al.*, 1970). (3) Aurintricarboxylic acid and pactamycin have the same effect on incorporation of f[^{35}S]-Met-tRNA$_f^{Met}$ and [^{35}S]Met-tRNA$_f^{Met}$ (Housman *et al.*, 1970) although, of course, normal incorporation from [^{35}S]Met-tRNA$_f$ is much less than that from the formylated derivative.

ATA and Pactamycin. Our principal result is that, at appropriate concentrations, both ATA and pactamycin selectively and totally block initiation of protein synthesis by extracts of rabbit reticulocytes, but do not affect completion of nascent chains. Incorporation by the initiator tRNAs fMet-tRNA$_f$ (Figure 1) or Met-tRNA$_f$ (Housman *et al.*, 1970) is totally blocked; incorporation of radioactive amino acids which are specific for internal positions of hemoglobin (Met-tRNA$_M^{Met}$) (Figure 2), [^{14}C]lysine, or [^{14}C]leucine (unpublished data) continues for 1 or 2 min at 25° and then ceases. During this period the polyribosomes are almost completely converted to monoribosomes (Figures 3 and 4) with the release of nascent hemoglobin chains (Figure 3). The time required for completion of existing chains (about 2 min at 25°; Figures 2 and 4) is in fair agreement with the estimate of Hunt *et al.* (1969) that, at 25°, it takes whole cells about 60–90 sec to synthesize one globin chain. Hence, our experiments do not eliminate the possibility that, at the lowest concentration of drug needed to completely block initiation, the rate of chain propagation is reduced by as much as a factor of 2.

These results are in agreement with earlier results on these drugs, mostly in bacterial systems. ATA specifically blocks attachment of phage RNA to *E. coli* ribosomes without affecting propagation of polypeptide chains (Grollman and Stewart, 1968; Webster and Zinder, 1968). Recently, Lebleu *et al.* (1970) showed that ATA also prevented the binding of globin mRNA to reticulocyte ribosomes. Likewise, pactamycin binds to the 30S ribosomal subunit of *E. coli* (Cohen *et al.*, 1969) and decreases the stability and rate of formation of the initiator complex with fMet-tRNA or *N*-acetylphenylalanyl-tRNA (Cohen *et al.*, 1969). Recently, MacDonald and Goldberg (1970) presented evidence, similar to that of Figure 2, that pactamycin blocks initiation of globin synthesis.

In the presence of either ATA and pactamycin, polyribosomes are converted predominantly to monoribosomes, and to a lesser extent to 40S and 60S subunits (Figures 3 and 4). This suggests that the drugs block either dissociation of ribosomes into subunits which are required for chain initiation or allow formation of an aberrant initiation complex (containing both subunits and presumably mRNA) by blocking some subsequent step.

We emphasize that concentrations of ATA or pactamycin twenty to forty times those used in Figures 1–3 do inhibit also elongation of nascent chains. This is seen both by reduced incorporation of radioactive amino acids into the nascent chains (Figure 4) and inhibition of ribosome movement along the mRNA (Figure 5). Siegelman (1970) has reported that such concentrations of ATA can block many steps in protein biosynthesis such as charging of tRNA and ribosome-dependent GTPase catalyzed by T and G factors. Likewise, Cohen and Goldberg (1967) reported that pactamycin also blocks attachment of peptidyl-tRNA to ribosomes; as transfer of peptidyl-tRNA from one ribosome binding site to another is an integral part of translocation, it is possible that higher levels of pactamycin also inhibit this reaction.

These findings emphasize the need for proper attention to concentration in applications of these drugs, especially to whole cells. For instance, Colombo *et al.* (1966) showed that addition of pactamycin to intact reticulocytes caused a disappearance of polyribosomes, but only after 15-min incubation; presumably the concentration of the drug inside the cell was either too low or too high to specifically block globin initiation.

Cycloheximide and Emetine. The major effects of these drugs appears to be on the propagation of the growing peptide chain. In studies of these drugs over a wide concentration range, there was no preferential inhibition of incorporation from the initiator tRNA, f[^{35}S]Met-tRNA$_f^{Met}$ (Figure 7). At low concentrations of the drugs the residual incorporation from [^{35}S]Met-tRNA$_M^{Met}$ was linear with time (Figures 6 and 7), a result which showed that those drugs do not preferentially block initiation of new chains while permitting completion of the existing nascent ones. That the drugs block ribosome movement along the mRNA is shown by the unchanged nature of the polysome profile after 3-min incubation at drug concentrations (a) where inhibition of amino acid incorporation is only partially blocked; (b) where synthesis is just totally abolished; and (c) at 20 times those concentrations (Figure 8). After 20-min incubation cycloheximide, at concentrations which inhibit protein synthesis 60–90%, causes a decrease in the level of ribosome subunits, and an increase in the amount of large polyribosomes (Figure 7). This result means that such concentrations affect the initiation of new globin chains much less than the movement of ribosomes along the mRNA, so that each mRNA becomes attached, on the average, to more ribosomes. This result is consistent with the recent finding (McKeehan and Hardesty, 1969) that cycloheximide specifically blocks the translocation reaction. Emetine has both a structural and functional similarity to cycloheximide (Grollman, 1966) and may also act preferentially at this step.

Inhibition by f2 RNA. The specific inhibition of initiation of hemoglobin synthesis by bacteriophage f2 RNA appears similar to the inhibition by synthetic homoribopolynucleotides observed by Hardesty *et al.* (1963). That formaldehyde-treated RNA is a better inhibitor than normal RNA may simply be a consequence of its more open secondary structure (Boedtker, 1967; Lodish, 1970) and availability of additional ribosome attachment sites (Lodish, 1970). We prefer to postpone any detailed discussion about the mechanism—and possible significance—of inhibition by viral RNA until we determine: (a) whether other viral RNAs show similar effects; (b) the

stage in hemoglobin initiation that is blocked; and (c) whether f2 RNA or formaldehyde-treated RNA is translated in reticulocyte extracts.

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